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(71) Applicant (for all designated States except US): ZENECA LIMITED [GB/GB]; 15 Stanhope Gate, London W1Y 6LN (GB).				MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).
(72) Inventors; and				
(75) Inventors/Applicants (for US only): JEPSON, Ian [GB/GB]; 31 Gringer Hill, Maidenhead, Berkshire SL6 7LY (GB). MARTINEZ, Alberto [GB/GB]; Ivy Cottage, Terrace Road South, Binfield, Berkshire RG42 4DS (GB). GREENLAND, Andrew, James [GB/GB]; Tree Tops, Kingswood Court, Braywick Road, Maidenhead, Berkshire SL6 1DA (GB).				
(74) Agents: ROBERTS, Alison, Christine et al.; Zeneca Agrochemicals, Intellectual Property Dept., Jealott's Hill Research Station, P.O. Box 3538, Bracknell, Berkshire RG42 6YA (GB).				

(54) Title: A GENE SWITCH COMPRISING AN ECDYSONE RECEPTOR

(57) Abstract

The invention relates to an insect steroid receptor protein which is capable of acting as a gene switch which is responsive to a chemical inducer enabling external control of the gene.

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A gene switch comprising an ecdysone receptor

The present invention relates to the identification and characterisation of insect steroid receptors from the Lepidoptera species *Heliothis virescens*, and the nucleic acid encoding therefor. The present invention also relates to the use of such receptors, and such nucleic acid, particularly, but not exclusively, in screening methods, and gene switches.

By gene switch we mean a gene sequence which is responsive to an applied exogenous chemical inducer enabling external control of expression of the gene controlled by said gene sequence.

Lipophilic hormones such as steroids induce changes in gene expression to elicit profound effects on growth, cellular differentiation, and homeostasis. These hormones recognise intracellular receptors that share a common modular structure consisting of three main functional domains: a variable amino terminal region that contains a transactivation domain, a DNA binding domain, and a ligand binding domain on the carboxyl side of the molecule. The DNA binding domain contains nine invariant cysteines, eight of which are involved in zinc coordination to form a two-finger structure. In the nucleus the hormone-receptor complex binds to specific enhancer-like sequences called hormone response elements (HREs) to modulate transcription of target genes.

The field of insect steroid research has undergone a revolution in the last three years as a result of the cloning and preliminary characterisation of the first steroid receptor member genes. These developments suggest the time is ripe to try to use this knowledge to improve our tools in the constant fight against insect pests. Most of the research carried out on the molecular biology of the steroid receptor superfamily has been on *Drosophila melanogaster* (Diptera), see for example International Patent Publication No WO91/13167, with some in *Manduca* and *Galleria* (Lepidoptera).

It has been three decades since 20-hydroxyecdysone was first isolated and shown to be involved in the regulation of development of insects. Since then work has been carried out to try to understand the pathway by which this small hydrophobic molecule regulates a number of activities. By the early 1970s, through the studies of Clever and Ashburner, it was clear that at least in the salivary glands of third instar *Drosophila* larvae, the application of ecdysone lead to the reproducible activation of over a hundred genes. The ecdysone receptor in this pathway is involved in the regulation of two classes of genes: a small class (early genes) which are induced by the ecdysone receptor and a large class (late genes) which are repressed by the ecdysone receptor. The early class of genes are thought to have two functions reciprocal to those of the ecdysone receptor; the repression of the early transcripts and the induction of late gene transcription. Members of the early genes so far isolated and characterised belong to the class of molecules with characteristics similar to known

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transcription factors. They are thus predicted to behave as expected by the model of ecdysone action (Ashburner, 1991). More recently, the early genes E74 and E75 have been shown to bind both types of ecdysone inducible genes (Thummel et al., 1990; Segraves and Hogness, 1991), thus supporting their proposed dual activities. It should be noted however, that the 5 activation of a hierarchy of genes is not limited to third instar larvae salivary glands, but that the response to the ecdysone peak at the end of larval life is observed in many other tissues, such as the imaginal disks (i.e. those tissues which metamorphose to adult structures) and other larval tissues which histolyse at the end of larval life (e.g. larval fat body). The model for ecdysone action as deduced by studying the third instar chromosome puffing may not apply 10 to the activation of ecdysone regulated genes in adults. In other words, the requirement for other factors in addition to the active ecdysone receptor must be satisfied for correct developmental expression (e.g. the *Drosophila* yolk protein gene expression in adults is under control of doublesex, the last gene in the sex determination gene hierarchy).

The ecdysone receptor and the early gene E75 belong to the steroid receptor 15 superfamily. Other *Drosophila* genes, including ultrspiracle, tailless, sevenup and FTZ-F1, also belong to this family. However, of all these genes only the ecdysone receptor is known to have a ligand, and thus the others are known as orphan receptors. Interestingly, despite the ultrspiracle protein ligand binding region sharing 49% identity with the vertebrate retinoic X receptor (RXR) ligand binding region (Oro et al., 1990), they do not share the 20 same ligand (i.e. the RXR ligand is 9-cis retinoic acid) (Heymann et al., 1992 and Mangelsdorf et al., 1992). All the *Drosophila* genes mentioned are involved in development, ultrspiracle for example, is required for embryonic and larval abdominal development. The protein products of these genes all fit the main features of the steroid receptor superfamily 25 (Evans, 1988; Green and Chambon, 1988, Beato, 1989) i.e. they have a variable N terminus region involved in ligand independent transactivation (Domains A and B), a highly conserved 66-68 amino acid region which is responsible for the binding of DNA at specific sites (Domain C), a hinge region thought to contain a nuclear translocation signal (Domain D), and a well conserved region containing the ligand binding region, transactivation sequences 30 and the dimerisation phase (Domain E). The last region, domain F, is also very variable and its function is unknown.

Steroid receptor action has been elucidated in considerable detail in vertebrate systems at both the cellular and molecular levels. In the absence of ligand, the receptor molecule resides in the cytoplasm where it is bound by Hsp90, Hsp70, and p59 to form the inactive complex (Evans, 1988). Upon binding of the ligand molecule by the receptor a conformational 35 change takes place which releases the Hsp90, Hsp70 and p59 molecules, while exposing the nuclear translocation signals in the receptor. The ligand dependent conformational change is seen in the ligand binding domain of both progesterone and retinoic acid receptors (Allan et

al., 1992a). This conformational change has been further characterised in the progesterone receptor and was found to be indispensable for gene transactivation (Allan et al., 1992b). Once inside the nucleus the receptor dimer binds to the receptor responsive element at a specific site on the DNA resulting in the activation or repression of a target gene. The 5 receptor responsive elements usually consist of degenerate direct repeats, with a spacer between 1 and 5 nucleotides, which are bound by a receptor dimer through the DNA binding region (Domain C).

Whereas some steroid hormone receptors are active as homodimers others act as heterodimers. For example, in vertebrates, the retinoic acid receptor (RAR) forms 10 heterodimers with the retinoic X receptor (RXR). RXR can also form heterodimers with the thyroid receptor, vitamin D receptor (Yu et al., 1991; Leid et al., 1992) and peroxisome activator receptor (Kliewer et al., 1992). Functionally the main difference between homodimers and heterodimers is increased specificity of binding to specific response 15 elements. This indicates that different pathways can be linked, co-ordinated and modulated, and more importantly this observation begins to explain the molecular basis of the pleotropic activity of retinoic acid in vertebrate development (Leid et al., 1992b). Similarly, the *Drosophila* ultraspirelce gene product was recently shown to be capable of forming heterodimers with retinoic acid, thyroid, vitamin D and peroxisome activator receptors and to stimulate the binding of these receptors to their target responsive elements (Yao et al., 1993). 20 More significantly, the ultraspirelce gene product has also been shown to form heterodimers with the ecdysone receptor, resulting in cooperative binding to the ecdysone response element and capable of rendering mammalian cells ecdysone responsive (Yao et al., 1992). The latter is of importance since transactivation of the ecdysone gene alone in mammalian cells fails to elicit an ecdysone response (Koelle et al., 1991), therefore suggesting that the ultraspirelce 25 gene product is an integral component of a functional ecdysone receptor (Yao et al., 1992). It is possible that the ultraspirelce product competes with other steroid receptors or factors to form heterodimers with the ecdysone receptor. Moreover it remains to be investigated if ultraspirelce is expressed in all tissues of the *Drosophila* larvae. Despite ultraspirelce being necessary to produce a functional ecdysone receptor, the mechanism by which this activation 30 takes place is as yet undetermined.

We have now isolated and characterised the ecdysone steroid receptor from *Heliothis virescens* (hereinafter HEcR). We have found that surprisingly unlike the *Drosophila* ecdysone steroid receptor (hereinafter DEcR), in reports to-date, HEcR can be induced by known non-steroidal inducers. It will be appreciated that this provides many advantages for 35 the system.

Steroids are difficult and expensive to make. In addition, the use of a non-steroid as the inducer allows the system to be used in agrochemical and pharmaceutical applications, not

least because it avoids application of a steroid which is already present in insects and/or mammals. For example, it would not be feasible to use a gene switch in a mammalian cell which was induced by a naturally occurring steroidal inducer. It will also be appreciated that for environmental reasons it is advantageous to avoid the use of steroids as inducers.

5 According to one aspect of the present invention there is provided DNA having the sequence shown in Seq ID No. 2, wherein Seq ID No 2 gives the sequence for the HEcR.

According to another aspect of the present invention there is provided DNA having part of the sequence shown in Seq ID No. 2, which encodes for the HEcR ligand binding domain.

10 According to another aspect of the present invention there is provided DNA having part of the sequence shown in Seq ID No. 2, which encodes for the HEcR DNA binding domain.

According to yet another aspect of the present invention there is provided DNA having part of the sequence shown in Seq ID No. 2, which encodes for the HEcR transactivation domain.

15 According to a further aspect of the present invention there is provided DNA having part of the sequence shown in Seq ID No. 2, which encodes for the HEcR hinge domain.

According to a still further aspect of the present invention there is provided DNA having part of the sequence shown in Seq ID No. 2, which encodes for the HEcR carboxy terminal region.

20 According to one aspect of the present invention there is provided DNA having the sequence shown in Seq ID No. 3, wherein Seq ID No 3 gives the sequence for the HEcR.

According to another aspect of the present invention there is provided DNA having part of the sequence shown in Seq ID No. 3, which encodes for the HEcR ligand binding domain.

25 According to another aspect of the present invention there is provided DNA having part of the sequence shown in Seq ID No. 3, which encodes for the HEcR DNA binding domain.

30 According to yet another aspect of the present invention there is provided DNA having part of the sequence shown in Seq ID No. 3, which encodes for the HEcR transactivation domain.

According to a further aspect of the present invention there is provided DNA having part of the sequence shown in Seq ID No. 3, which encodes for the HEcR hinge domain.

35 According to a still further aspect of the present invention there is provided DNA having part of the sequence shown in Seq ID No. 3, which encodes for the HEcR carboxy terminal region.

According to one aspect of the present invention there is provided DNA having the sequence shown in Seq ID No. 4, wherein Seq ID No 4 gives the sequence for the HEcR.

According to another aspect of the present invention there is provided DNA having part of the sequence shown in Seq ID No. 4, which encodes for the HEcR ligand binding domain.

According to another aspect of the present invention there is provided DNA having part of the sequence shown in Seq ID No. 4, which encodes for the HEcR DNA binding domain.

According to yet another aspect of the present invention there is provided DNA having part of the sequence shown in Seq ID No. 4, which encodes for the HEcR transactivation domain.

According to a further aspect of the present invention there is provided DNA having part of the sequence shown in Seq ID No. 4, which encodes for the HEcR hinge domain.

According to a still further aspect of the present invention there is provided DNA having part of the sequence shown in Seq ID No. 4, which encodes for the HEcR carboxy terminal region.

As mentioned above, steroid receptors are eukaryotic transcriptional regulatory factors which, in response to the binding of the steroid hormone, are believed to bind to specific DNA elements and activate transcription. The steroid receptor can be divided into six regions, designated A to F, using alignment techniques based on shared homology with other members of the steroid hormone receptor superfamily. Krust et al identified two main regions in the receptor, C and E. Region C is hydrophilic and is unusual in its high content in cysteine, lysine and arginine. It corresponds to a DNA-binding domain, sometimes referred to as the "zinc finger". It is the DNA binding domain which binds to the upstream DNA of the responsive gene. Such upstream DNA is known as the hormone response element or HRE for short. Region E is hydrophobic and is identified as the hormone (or ligand) binding domain. Region E can be further subdivided into regions E1, E2 and E3.

The region D, which separates domains C and E is highly hydrophobic and is flexible. It is believed that communication between domains E and C involves direct contact between them through region D, which provides a hinge between the two domains. Region D is therefore referred to as the hinge domain.

The mechanism of the receptor appears to require it to interact with some element(s) of the transcription machinery over and above its interactions with the hormone and the hormone response element. N-terminal regions A and B perform such a function and are jointly known as the transactivation domain. The carboxy terminal region is designated F.

The domain boundaries of the HEcR can be defined as follows:

DOMAIN	INTERVALS	
	base pairs	amino acids
Transactivating (A/B)	114-600	1-162
DNA Binding (C)	601-798	163-228
Hinge (D)	799-1091	229-326
Ligand Binding (E)	1092-1757	327-545
C-Terminal End (F)	1758-1844	546-577

The DNA binding domain is very well defined and is 66 amino acids long, thus providing good boundaries. The above intervals have been defined using the multiple alignment for the ecdysone receptors (Figure 5).

The present invention also includes DNA which shows homology to the sequences of the present invention. Typically homology is shown when 60% or more of the nucleotides are common, more typically 65%, preferably 70%, more preferably 75%, even more preferably 80% or 85%, especially preferred are 90%, 95%, 98% or 99% or more homology.

The present invention also includes DNA which hybridises to the DNA of the present invention and which codes for at least part of the *Heliothis* ecdysone receptor transactivation domain, DNA binding domain, hinge domain, ligand binding domain and/or carboxy terminal region. Preferably such hybridisation occurs at, or between, low and high stringency conditions. In general terms, low stringency conditions can be defined as 3 x SSC at about ambient temperature to about 65°C, and high stringency conditions as 0.1 x SSC at about 65°C. SSC is the name of a buffer of 0.15M NaCl, 0.015M trisodium citrate. 3 x SSC is three time as strong as SSC and so on.

The present invention further includes DNA which is degenerate as a result of the genetic code to the DNA of the present invention and which codes for a polypeptide which is at least part of the *Heliothis* ecdysone receptor transactivation domain, DNA binding domain, hinge domain, ligand binding domain and/or carboxy terminal region.

The DNA of the present invention may be cDNA or DNA which is in an isolated form.

According to another aspect of the present invention there is provided a polypeptide comprising the *Heliothis* ecdysone receptor or a fragment thereof, wherein said polypeptide is substantially free from other proteins with which it is ordinarily associated, and which is coded for by any of the DNA of the present invention.

According to another aspect of the present invention there is provided a polypeptide which has the amino acid sequence of Seq ID No. 4 or any allelic variant or derivative thereof, wherein Seq ID No. 4 gives the amino acid sequence of the HEcR polypeptide.

According to another aspect of the present invention there is provided a polypeptide which has part of the amino acid sequence of Seq ID No. 4 or any allelic variant or derivative thereof, which sequence provides the HEcR ligand binding domain.

5 According to another aspect of the present invention there is provided a polypeptide which has part of the amino acid sequence of Seq ID No. 4 or any allelic variant or derivative thereof, which sequence provides the HEcR DNA binding domain.

According to yet another aspect of the present invention there is provided a polypeptide which has part of the amino acid sequence of Seq ID No. 4 or any allelic variant or derivative thereof, which sequence provides the HEcR transactivation domain.

10 According to a further aspect of the present invention there is provided a polypeptide which has the amino acid sequence of a part of Seq ID No. 4 or any allelic variant or derivative thereof, which sequence provides the HEcR hinge domain.

15 According to a still further aspect of the present invention there is provided a polypeptide which has the amino acid sequence of a part of Seq ID No. 4 or any allelic variant or derivative thereof, which sequence provides the HEcR carboxy terminal region.

For the avoidance of doubt, spliced variants of the amino acid sequences of the present invention are included in the present invention.

20 Preferably, said derivative is a homologous variant which has conservative amino acid changes. By conservation amino acid changes we mean replacing an amino acid from one of the amino acid groups, namely hydrophobic, polar, acidic or basic, with an amino acid from within the same group. An examples of such a change is the replacement of valine by methionine and vice versa.

25 According to another aspect of the present invention there is provided a fusion polypeptide comprising at least one of the polypeptides of the present invention functionally linked to an appropriate non-*Heliothis* ecdysone receptor domain(s).

According to an especially preferred embodiment of the present invention the HEcR ligand binding domain of the present invention is fused to a DNA binding domain and a transactivation domain.

30 According to another embodiment of the present invention the DNA binding domain is fused to a ligand binding domain and a transactivation domain.

According to yet another embodiment of the present invention the transactivation domain is fused to a ligand binding domain and a DNA binding domain.

The present invention also provides recombinant DNA encoding for these fused polypeptides.

35 According to an especially preferred embodiment of the present invention there is provided recombinant nucleic acid comprising a DNA sequence encoding the HEcR ligand

binding domain functionally linked to DNA encoding the DNA binding domain and transactivation domain from a glucocorticoid receptor.

According to yet another aspect of the present invention there is provided recombinant nucleic acid comprising a DNA sequence comprising a reporter gene operably linked to a promoter sequence and a hormone response element which hormone response element is responsive to the DNA bonding domain encoded by the DNA of the present invention.

According to another aspect of the present invention there is provided a construct transformed with nucleic acid, recombinant DNA, a polypeptide or a fusion polypeptide of the present invention. Such constructs include plasmids and phages suitable for transforming a cell of interest. Such constructs will be well known to those skilled in the art.

According to another aspect of the present invention there is provided a cell transformed with nucleic acid, recombinant DNA, a polypeptide, or a fusion polypeptide of the present invention.

15 Preferably the cell is a plant, fungus or mammalian cell.

For the avoidance of doubt fungus includes yeast.

The present invention therefore provides a gene switch which is operably linked to a foreign gene or a series of foreign genes whereby expression of said foreign gene or said series of foreign genes may be controlled by application of an effective exogenous inducer.

20 Analogs of ecdysone, such as Muristerone A, are found in plants and disrupt the development of insects. It is therefore proposed that the receptor of the present invention can be used in plants transformed therewith as an insect control mechanism. The production of the insect-damaging product being controlled by an exogenous inducer. The insect-damaging product can be ecdysone or another suitable protein.

25 The first non-steroidal ecdysteroid agonists, dibenzoyl hydrazines, typified by RH-5849 [1,2-dibenzoyl, 1-tert-butyl hydrazide], which is commercially available as an insecticide from Rohm and Haas, were described back in 1988. Another commercially available compound in this series is RH-5992 [tebufenozone, 3,5-dimethylbenzoic acid 1-(1,1-dimethylethyl)-2(4-ethylbenzoyl) hydrazide]. These compounds mimic

30 20-hydroxyecdysone (20E) in both *Manduca sexta* and *Drosophila melanogaster*. These compounds have the advantage that they have the potential to control insects using ecdysteroid agonists which are non-steroidal. Further Examples of such dibenzoyl hydrazines are given in US Patent No. 5,117,057 to Rohm and Haas, and Oikawa et al, Pestic Sci, 41, 139-148 (1994). However, it will be appreciated that any inducer of the gene switch of the

35 present invention, whether steroid or non-steroidal, and which is currently or becomes available, may be used.

The gene switch of the present invention, then, when linked to an exogenous or foreign gene and introduced into a plant by transformation, provides a means for the external regulation of expression of that foreign gene. The method employed for transformation of the plant cells is not especially germane to this invention and any method suitable for the target plant may be employed. Transgenic plants are obtained by regeneration from the transformed cells. Numerous transformation procedures are known from the literature such as agroinfection using *Agrobacterium tumefaciens* or its Ti plasmid, electroporation, microinjection or plants cells and protoplasts, microprojectile transformation, to mention but a few. Reference may be made to the literature for full details of the known methods.

Neither is the plant species into which the chemically inducible sequence is inserted particularly germane to the invention. Dicotyledonous and monocotyledonous plants can be transformed. This invention may be applied to any plant for which transformation techniques are, or become, available. The present invention can therefore be used to control gene expression in a variety of genetically modified plants, including field crops such as canola, sunflower, tobacco, sugarbeet, and cotton; cereals such as wheat, barley, rice, maize, and sorghum; fruit such as tomatoes, mangoes, peaches, apples, pears, strawberries, bananas and melons; and vegetables such as carrot, lettuce, cabbage and onion. The switch is also suitable for use in a variety of tissues, including roots, leaves, stems and reproductive tissues.

In a particularly preferred embodiment of the present invention, the gene switch of the present invention is used to control expression of genes which confer resistance herbicide resistance and/or insect tolerance to plants.

Recent advances in plant biotechnology have resulted in the generation of transgenic plants resistant to herbicide application, and transgenic plants resistant to insects. Herbicide tolerance has been achieved using a range of different transgenic strategies. One well documented example in the herbicide field is the use the bacterial xenobiotic detoxifying gene phosphinothricin acetyl transferase (PAT) from *Streptomyces hygroscopicus*. Mutated genes of plant origin, for example the altered target site gene encoding acetolactate synthase (ALS) from *Arabidopsis*, have been successfully utilised to generate transgenic plants resistant to herbicide application. The PAT and ALS genes have been expressed under the control of strong constitutive promoter. In the field of insecticides, the most common example to-date is the use of the Bt gene.

We propose a system where genes conferring herbicide and/or insect tolerance would be expressed in an inducible manner dependent upon application of a specific activating chemical. This approach has a number of benefits for the farmer, including the following:

1. Inducible control of herbicide and/or insect tolerance would alleviate any risk of yield penalties associated with high levels of constitutive expression of herbicide and/or insect resistance genes. This may be a particular problem as early stages of growth

where high levels of transgene product may directly interfere with normal development. Alternatively high levels of expression of herbicide and/or insect resistance genes may cause a metabolic drain for plant resources.

2. The expression of herbicide resistance genes in an inducible manner allows the herbicide in question to be used to control volunteers if the activating chemical is omitted during treatment.
5. The use of an inducible promoter to drive herbicide and/or insect resistance genes will reduce the risk of resistance becoming a major problem. If resistance genes were passed onto weed species from related crops, control could still be achieved with the herbicide in the absence of inducing chemical. This would particularly be relevant if the tolerance gene confirmed resistance to a total vegetative control herbicide which would be used (with no inducing chemical) prior to sowing the crop and potentially after the crop has been harvested. For example, it can be envisaged that herbicide resistance cereals, such as wheat, might outcross into the weed wild oats, thus conferring herbicide resistance to this already troublesome weed. A further example is that the inducible expression of herbicide resistance in sugar beet will reduce the risk of wild sugar beet becoming a problem. Similarly, in the field of insect control, insect resistance may well become a problem if the tolerance gene is constitutively expressed. The use of an inducible promoter will allow a greater range of insect resistance control mechanisms to be employed.
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- 15
- 20

This strategy of inducible expression of herbicide resistance can be achieved with a pre-spray of chemical activator or in the case of slow acting herbicides, for example N-phosphonomethyl-glycine (commonly known as glyphosate), the chemical inducer can be added as a tank mix simultaneously with the herbicide. Similar strategies can be employed for insect control.

This strategy can be adopted for any resistance conferring gene/ corresponding herbicide combination, which is, or becomes, available. For example, the gene switch of the present invention can be used with:

1. Maize glutathione S-transferase (GST-27) gene (see our International Patent Publication No WO90/08826), which confers resistance to chloroacetanilide herbicides such as acetochlor, metolachlor and alachlor.
- 30
2. Phosphinotricin acetyl transferase (PAT), which confers resistance to the herbicide commonly known as glufosinate.
3. Acetolactate synthase gene mutants from maize (see our International Patent Publication No WO90/14000) and other genes, which confer resistance to sulphonyl urea and imadazolinones.
- 35

4: Genes which confer resistance to glyphosate. Such genes include the glyphosate oxidoreductase gene (GOX) (see International Patent Publication No. WO92/00377); genes which encode for 5-enolpyruvyl-3-phosphoshikimic acid synthase (EPSPS), including Class I and Class II EPSPS, genes which encode for mutant EPSPS, and genes which encode for EPSPS fusion peptides such as that comprised of a chloroplast transit peptide and EPSPS (see for example EP 218 571, EP 293 358, WO91/04323, WO92/04449 and WO92/06201); and genes which are involved in the expression of CPLyase.

5 Similarly, the strategy of inducible expression of insect resistance can be adopted for
10 any tolerance conferring gene which is, or becomes, available.

The gene switch of the present invention can also be used to controlled expression of foreign proteins in yeast and mammalian cells. Many heterologous proteins for many applications are produced by expression in genetically engineered bacteria, yeast cells and other eucaryotic cells such as mammalian cells.

15 As well as the obvious advantage in providing control over the expression of foreign genes in such cells, the switch of the present invention provides a further advantage in yeasts and mammalian cells where accumulation of large quantities of an heterologous protein can damage the cells, or where the heterologous protein is damaging such that expression for short periods of time is required in order to maintain the viability of the cells.

20 Such an inducible system also has applicability in gene therapy allowing the timing of expression of the therapeutic gene to be controlled. The present invention is therefore not only applicable to transformed mammalian cells but also to mammals *per se*.

25 A further advantage of the inducible system of the present invention in mammalian cells is that, because it is derived from a insect, there is less chance of it being effected by inducers which effect the natural mammalian steroid receptors.

30 In another aspect of the present invention the gene switch is used to switch on genes which produce potentially damaging or lethal proteins. Such a system can be employed in the treatment of cancer in which cells are transformed with genes which express proteins which are lethal to the cancer. The timing of the action of such proteins on the cancer cells can be controlled using the switch of the present invention.

The gene switch of the present invention can also be used to switch genes off as well as on. This is useful in disease models. In such a model the cell is allowed to grow before a specific gene(s) is switched off using the present invention. Such a model facilitates the study of the effect of a specific gene(s).

35 Again the method for producing such transgenic cells is not particularly germane to the present invention and any method suitable for the target cell may be used; such methods are known in the art, including cell specific transformation.

As previously mentioned, modulation of gene expression in the system appears in response to the binding of the HEcR to a specific control, or regulatory, DNA element. A schematic representation of the HEcR gene switch is shown in Figure 6. For ease of reference, the schematic representation only shows three main domains of the HEcR, namely 5 the transactivation domain, DNA binding domain and the ligand binding domain. Binding of a ligand to the ligand binding domain enables the DNA binding domain to bind to the HRE resulting in expression (or indeed repression) of a target gene.

The gene switch of the present invention can therefore be seen as having two components. The first component comprising the HEcR and a second component comprising 10 an appropriate HRE and the target gene. In practice, the switch may conveniently take the form of one or two sequences of DNA. At least part of the one sequence, or one sequence of the pair, encoding the HEcR protein. Alternatively, the nucleic acid encoding the HEcR can be replaced by the protein/ polypeptide itself.

Not only does the switch of the present invention have two components, but also one 15 or more of the domains of the receptor can be varied producing a chimeric gene switch. The switch of the present invention is very flexible and different combinations can be used in order to vary the result/to optimise the system. The only requirement in such chimeric systems is that the DNA binding domain should bind to the hormone response element in order to produce the desired effect.

20 The glucocorticoid steroid receptor is well characterised and has been found to work well in plants. A further advantage of this receptor is that it functions as a homodimer. This means that there is no need to express a second protein such as the ultraspiracle in order to produce a functional receptor. The problem with the glucocorticoid steroid receptor is that ligands used to activate it are not compatible with agronomic practice.

25 In a preferred aspect of the present invention the receptor comprises glucocorticoid receptor DNA binding and transactivation domains with a *Heliothis* ligand binding domain according to the present invention. The response unit preferably comprising the glucocorticoid hormone response element and the desired effect gene. In the Examples, for convenience, this effect gene took the form of a reporter gene. However, in non-test or non- 30 screen situations the gene will be the gene which produces the desired effect, for example produces the desired protein. This protein may be a natural or exogenous protein. It will be appreciated that this chimeric switch combines the best features of the glucocorticoid system, whilst overcoming the disadvantage of only being inducible by a steroid.

In another preferred embodiment, the *Heliothis* ligand binding domain is changed, 35 and preferably replaced with a non-*Heliothis* ecdysone receptor ligand binding domain. For example, we have isolated suitable sequences from *Spodoptera exigua*.

Thus, according to another aspect of the present invention there is provided DNA having the sequence shown in Seq ID No. 6.

According to another aspect of the present invention there is provided DNA having part of the sequence shown in Seq ID No. 6, which encodes for the *Spodoptera* ecdysone ligand binding domain.
5

According to another aspect of the present invention there is provided DNA having part of the sequence shown in Seq ID No. 6, which encodes for the *Spodoptera* ecdysone hinge domain.

The present invention also provides the polypeptides coded for by the above DNA
10 sequences of Seq ID No. 6.

A further advantage with such chimeric systems is that they allow you to choose the promoter which is used to drive the effector gene according to the desired end result. For example, placing the foreign gene under the control of a cell specific promoter can be particularly advantageous in circumstances where you wish to control not only the timing of
15 expression, but also which cells expression occurs in. Such a double control can be particularly important in the areas of gene therapy and the use of cytotoxic proteins.

Changing the promoter also enables gene expression to be up- or down-regulated as desired.

Any convenient promoter can be used in the present invention, and many are known in
20 the art.

Any convenient transactivation domain may also be used. The transactivation domain VP16 is a strong activator from Genentech Inc., and is commonly used when expressing glucocorticoid receptor in plants. Other transactivation domains derived for example from plants or yeast may be employed.

In a preferred embodiment of the present invention, the DNA binding domain is the glucocorticoid DNA binding domain. This domain is commonly a human glucocorticoid receptor DNA binding domain. However, the domain can be obtained from any other convenient source, for example, rats.
25

According to another aspect of the present invention there is provided a method of selecting compounds capable of being bound to an insect steroid receptor superfamily member comprising screening compounds for binding to a polypeptide or fusion polypeptide of the present invention, and selecting said compounds exhibiting said binding.
30

According to another aspect of the present invention there is provided a compound selected using the method of the present invention.

According to another aspect of the present invention there is provided an agricultural or pharmaceutical composition comprising the compound of the present invention.
35

According to yet another aspect of the present invention there is provided the use of the compound of the present invention as a pesticide, pharmaceutical and/or inducer of the switch. It will be appreciated that such inducers may well be useful as insecticides in themselves.

5 According to a further aspect of the present invention there is provided a method of producing a protein or peptide or polypeptide comprising introducing into a cell of the present invention, a compound which binds to the ligand binding domain in said cell.

Various preferred features and embodiments of the present invention will now be described by way of non-limiting example with reference to the accompanying examples and 10 figures, in which figures:

Figure 1 (Sequence ID No. 1) shows the DNA sequence amplified from first strand cDNA made from mRNA isolated from *Heliothis virescens* Fourth instar larvae. The underlined sequences refer to the position of the degenerate oligonucleotides. At the 5' end the sequence matches that of the oligonucleotide while at the 3' end 12 nucleotides of the 15 original oligonucleotide are observed;

Figure 2 (Sequence ID No. 2) shows the DNA sequence contained within the clone pSK19R isolated from a random primed cDNA *Heliothis virescens* library; Sequence is flanked by EcoRI sites;

Figure 3 (Sequence ID No. 3) shows the DNA sequence contained within the clone 20 pSK16.1 isolated from a random primed cDNA *Heliothis virescens* library ;

Figure 4 (Sequence ID No. 4) DNA sequence of 5'RACE products (in bold) fused to sequence of clone pSK16.1. The ORF (open reading frame) giving rise to the *Heliothis virescens* ecdysone receptor protein sequence is shown under the corresponding DNA sequence;

25 Figure 5 (Sequence ID No. 5) shows the protein sequence alignment of the ecdysone receptors DmEcR (*Drosophila melanogaster*), CtEcR (*Chironomus tentans*), BmEcR (*Bombyx mori*), MsEcR (*Manduca sexta*), AaEcR (*Aedes aegypti*) and HvEcR (*Heliothis virescens*). “*” indicates conserved amino acid residue. “.” indicates a conservative amino acid exchange;

30 Figure 6 shows a model of an embodiment of the glucocorticoid/*Heliothis* ecdysone chimeric receptor useable as a gene switch;

Figure 7 shows a plasmid map of the clone pcDNA319R. The three other mammalian expression vectors were constructed in the same way and look similar but for the size of the insert;

35 Figure 8 shows a plasmid map of the reporter construct used to analyse the activity of the *Heliothis virescens* ecdysone receptor;

Figure 9 is a graph which shows the effect of Muristerone A and RH5992 in reporter activity in HEK293 cells co-transfected with pcDNA3H3KHEcR alone (filled bars) or with α RXR (stripped bars);

5 Figure 10 shows a plasmid map of the Maize expression vector containing the Glucocorticoid receptor (HG1 or pMF6HG1PAT);

Figure 11 shows a plasmid map of the maize expression vector containing the chimeric glucocorticoid/*Drosophila* ecdysone receptor pMF6GREcRS;

10 Figure 12 shows a plasmid map of the maize expression vector containing the chimeric glucocorticoid/*Heliothis* ecdysone receptor pMF6GRHEcR;

10 Figure 13 shows a plasmid map of the plant reporter Plasmid containing the glucocorticoid response elements fused to the -60 S35CaMV promoter fused to GUS, p221.9GRE6;

15 Figure 14 shows a plasmid map of the plant reporter plasmid containing the glucocorticoid response elements fused to the -46 S35CaMV promoter fused to GUS, p221.10GRE6;

Figure 15 shows a graph showing the effect of Muristerone A and Dexamethasone in Maize AXB protoplasts transformed with pMF6HG1PAT (GR) and p221.9GRE6 (reporter);

20 Figure 16 shows a graph showing the effect of Muristerone A and Dexamethasone in Maize AXB protoplasts transformed with pMF6GREcRS (effector) and p221.9GRE6 (reporter);

Figure 17 shows a graph showing the effect of Muristerone A and Dexamethasone in Maize AXB protoplasts transformed with pMF6GRHEcR (effector) and p221.9GRE6 (reporter);

25 Figure 18 shows a graph showing the effect of RH5849 in Maize AXB protoplasts transformed with pMF6GREcRS (effector) and p221.9GRE6 (reporter);

Figure 19 shows a graph showing the effect of RH5992 in Maize AXB protoplasts transformed with pMF6GREcRS (effector) and p221.9GRE6 (reporter);

Figure 20 shows a graph showing the effect of RH5992 in Maize AXB protoplasts transformed with pMF6GRHEcR (effector) and p221.9GRE6 (reporter);

30 Figure 21 shows a graph which shows the dose response effect of RH5992 in Maize AXB protoplasts transformed with pMF6GRHEcR (effector) and p221.9GRE6 (reporter);

Figure 22 shows a plasmid map of the tobacco expression vector containing the chimeric glucocorticoid/ *Drosophila* ecdysone receptor, pMF7GREcRS;

35 Figure 23 shows a plasmid map of the tobacco expression vector containing the chimeric glucocorticoid/ *Heliothis* ecdysone receptor, pMF7GRHEcR;

Figure 24 shows a graph which shows the effect of RH5992 in Tobacco mesophyll protoplasts transformed with pMF6GRHEcR (Effector) and p221.9GRE6 (reporter);

Figure 25 shows a plasmid map of the mammalian expression vector containing the chimeric glucocorticoid/*Heliothis* ecdysone receptor, pcDNA3GRHEcR;

Figure 26 shows a plasmid map of the reporter plasmid pSWGRE4;

Figure 27 shows a graph which shows a RH5992 dose response curve of CHO cells 5 transfected with pcDNA3GRHEcR and pSWGRE4;

Figure 28 shows a graph which shows the effect of Muristerone A and RH5992 on HEK293 cells co-transfected with pcDNA3GRHEcR and pSWGRE4;

Figure 29 shows a plasmid map of the binary vector ES1;

Figure 30 shows a plasmid map of the binary vector ES2;

10 Figure 31 shows a plasmid map of the binary vector ES3;

Figure 32 shows a plasmid map of the binary vector ES4;

Figure 33 shows a plasmid map of the effector construct TEV-B112 made to express the HEcR ligand binding domain in yeast;

Figure 34 shows a plasmid map of the effector construct TEV8 made to express the 15 HEcR ligand binding domain in yeast;

Figure 35 shows a plasmid map of the effector construct TEVVP16-3 made to express the HEcR ligand binding domain in yeast;

Figure 36 shows a plasmid map of the mammalian expression vector containing the chimeric glucocorticoid VP16/*Heliothis* ecdysone receptor, pcDNA3GRVP16HEcR;

20 Figure 37 shows a plasmid map of the maize expression vector containing the chimeric glucocorticoid VP16/*Heliothis* ecdysone receptor, pMF6GRVP16HEcR;

Figure 38 shows a plasmid map of the maize expression vector containing the chimeric glucocorticoid VP16/*Heliothis* ecdysone receptor, pMF7GRVP16HEcR;

Figure 39 shows a graph which shows the effect of RH5992 in Maize AXB 25 protoplasts transformed with pMF6GRVP16HEcR (effector) and p221.9GRE6 (reporter);

Figure 40 (Sequence ID No. 6) shows the DNA sequence of the hinge and ligand binding domains of the *Spodoptera exigua* ecdysone receptor;

Figure 41 (Sequence ID No. 7) shows the protein sequence alignment of the *Heliothis* 19R and *Spodoptera* SEcR *Taq* clone hinge and ligand binding domains. "*" indicates 30 conserved amino acid residue. ":" indicates a conservative amino acid exchange;

Figure 42 shows a graph which shows the effect of RH5992 on Tobacco mesophyll protoplasts transformed with pMF7GRHEcR (effector) and either p221.9GRE6 (Horizontal strips) or p221.10GRE6 (vertical strips).

Example I - Cloning of the *Heliothis* Ecdysone Receptor

A. Probe generation

5 The rational behind the generation of the probe to isolate *Heliothis* homologues to
the steroid/thyroid receptor superfamily members was based on comparing the sequences of
developmentally regulated steroid/thyroid receptor superfamily members. The sequences
available showed a highly conserved motif within the DNA binding domain of the RAR and
THR (thyroid) receptors. The motifs were used to design degenerate oligonucleotides for
10 PCR amplification of sequences derived from cDNA template produced from tissue expected
to express developmentally regulated steroid/thyroid receptor superfamily members (ie. larval
tissues).

The sense oligonucleotide is based on the peptide sequence CEGCKGFF which at the DNA level yields an oligonucleotide with degeneracy of 32 as shown below :

15

ZnFA5' 5' TGC GAG GGI TGC AAG GAI TTC TT 3'
 T A T A T

The antisense oligonucleotide is based on the reverse complement nucleotide sequence derived from the peptide:

20

CQECRLKK

S R

for which four sets of degenerate oligos were made. Namely:

ZnFA3' 5' TTC TTI AGI CGG CAC TCT TGG CA 3'
T A T C A

ZnFB3' 5' TTC TTI AAI CGG CAC TCT TGG CA 3'
T A T G A

30

ZnFC3' 5' TTC TTI AGI CTG CAC TCT TGG CA 3'
T G A C T G C A T G C A

35

The PCR amplification was carried out using a randomly primed cDNA library made from mRNA isolated from 4th and 5th instar *Heliothis virescens* larvae. The amplification

was performed using 10^8 pfus (plaque forming units) in 50mM KCl, 20mM Tris HCl pH 8.4, 15mM MgCl₂, 200mM dNTPs (an equimolar mixture of dCTP, dATP, dGTP and dTTP), 100ng of ZnFA5' and ZnF3' mixture. The conditions used in the reaction followed the hot start protocol whereby the reaction mixture was heated to 94°C for 5 minutes after which 1
5 U of Taq polymerase was added and the reaction allowed to continue for 35 cycles of 93°C for 50 seconds, 40°C for 1 minute and 73°C for 1 minute 30 seconds. The PCR products were fractionated on a 2%(w/v) agarose gel and the fragment migrating between 100 and 200bp markers was isolated and subcloned into the vector pCRII (Invitrogen). The sequence of the insert was determined using Sequenase (USB).

10 The resulting sequence was translated and a database search carried out. The search recovered sequences matching to the DNA binding domain of the *Drosophila* ecdysone receptor, retinoic acid receptor and the thyroid receptor. Thus, the sequence of the insert in this plasmid, designated pCRIIZnf, is a *Heliothis* ecdysone cognate sequence (Figure 1) and was used to screen a cDNA library in order to isolate the complete open reading frame.

15

B. Library screening

The randomly primed cDNA 4th/5th Instar *Heliothis virescens* library was plated and replicate filter made from the plates. The number of plaques plated was 500,000. The insert fragment of pCRIIZnf was reamplified and 50ng were end labelled using T4 Polynucleotide Kinase (as described in Sambrook et al 1990).

20 The filter were prehybridised using 0.25%(w/v) Marvel, 5 X SSPE and 0.1%(w/v) SDS at 42°C for 4 hours. The solution in the filters was then replaced with fresh solution and the denatured probe added. The hybridisation was carried out overnight at 42°C after which the filter were washed in 6 X SSC + 0.1%(w/v) SDS at 42°C followed by another wash at 25 55°C. The filter were exposed to X-ray film (Kodak) for 48 hours before processing.

25 The developed film indicated the presence of one strong positive signal which was plaque purified and further characterised. The lambda ZAP II phage was in vivo excised (see Stratagene Manual) and the sequence determined of the resulting plasmid DNA. The clone known as pSK19R (or 19R) contained a 1.933kb cDNA fragment with an open reading frame of 467 amino acids (Figure 2). pSK19R was deposited with the NCIMB on 20 June 1995 and has been accorded the deposit No NCIMB 40743.

30 Further analysis of pSK19R revealed that a 340 bp EcoRI fragment mapping at the 5' end of pSK19R has strong and significant similarities to a *Drosophila* cDNA encoding glyceraldehyde-3-phosphate dehydrogenase. In order to isolate the correct 5'end sequence belonging to *Heliothis*, the random primed library was re-screened using a probe containing the 5'end of the pSK19R belonging to *Heliothis* ecdysone receptor. The probe was made by PCR using the sense oligonucleotide HecRH3C (5' aat tag ctccaccatgcccgttaccaatgccaccgaca

3') and antisense oligonucleotide HecrNdeI (5' cttcaaccgacactcctgac 3'). The PCR was carried out as described by Hirst et al., 1992) where the amount of radioisotope used in the labelling was 50uCi of a ³²P-dCTP and the PCR was cycled for 1 minute at 94°C, 1 minute at 60°C and 1 minute at 72°C for 19 cycles. The resulting 353bp radio labelled DNA fragment
5 was denatured and added to prehybridised filters as described for the isolation of pSK19R. The library filters were made from 15 plates each containing 50000 pfus. The library filters were hybridised at 65°C and washed in 3XSSPE + 0.1%SDS at 65°C twice for 30 minutes each. The filters were further washed with 1XSSPE + 0.1%SDS for 30 minutes and exposed to X-ray film (Kodak) overnight. The film was developed and 16 putative positive plaques
10 were picked. The plaques were re-plated and hybridised under the exact same conditions as the primary screen resulting in only one strong positive. The strong positive was consistently recognised by the probe and was plaque purified and *in vivo* excised. The resulting plasmid pSK16.1 was sequenced (Seq 1D3) which revealed that the 5' end of the clone extended by 205 bp and at the 3' end by 653 bp and resulting in a DNA insert of 2.5 kb. Conceptual
15 translation of the 205 bp yielded 73 amino acids with high similarity to the *Drosophila*, *Aedes aegypti*, *Manduca* and *Bombyx* sequences of the ecdysone receptor B1 isoform. However, the whole of the 5' end sequence is not complete since a Methionine start site was not found with a stop codon in frame 5' of the methionine. In order to isolate the remainder of the 5'
20 end coding sequences a 5'RACE protocol (Rapid Amplification of cDNA Ends) was carried out using the BRL-GIBCO 5'RACE Kit. Two types of cDNA were synthesised where the first one used a specific oligonucleotide :
25 16PCR2A 5' cagctccaggccggcgatctcg3'

and the second type used random hexamers (oligonucleotide containing 6 random nucleotides). Each cDNA was PCR amplified using the oligonucleotides anchor primer :
BRL-GIBCO 5' cuacuacuacuaggccacgcgtcgactagtacgggiigggiigggiig 3'
and 16PCR2A and cycled for 1 minute at 94°C, 1 minute at 60°C and 1 minute at 72°C for 35 cycles. The reaction conditions were 20mM Tris-HCl (pH8.4), 50mM KCl, 1.5mM MgCl₂, 400nM of each anchor and 16PCR2A primers, 200mM dNTPs (dATP,dCTP,dGTP and dTTP) and 0.02 U/ml *Taq* DNA polymerase. Dilutions of 1:50 of the first PCR reactions
30 were made and 1ml was use in a second PCR with oligonucleotides UAP :
(Universal Amplification Primer 5' caucaucauggccacgcgtcgactagtac 3')
and 16RACE2 :
(5' acgtcacccatcgagacgagcttccattc 3').

The conditions and cycling were the same as those followed for the first PCR.
35 Samples of each PCR were run and a Southern blot carried out which was probed with a 5' specific primer :
(16PCR1 5' cgctggataacaacggaccattc 3').

This primer is specific for the 5' most sequence of pSK16.1 and was hybridised at 55°C using the standard hybridisation buffer. The filter was washed at 55°C 3 times in 3XSSPE + 0.1%SDS and exposed to X-ray film for up to 6 hours. The developed film revealed bands recognised by the oligonucleotide migrating at 100bp and 500bp (relative to the markers). A sample of the PCR reaction (4 in total) was cloned into the pCRII vector in the TA cloning kit (Invitrogen). Analysis of 15 clones from 4 independent PCRs yielded sequence upstream of pSK16.1 (Figure 4).

Translation of the ORF results in a 575 amino acid protein with high similarity in the DNA and ligand binding domains when compared to the ecdysone receptor sequences of 10 *Drosophila*, *Aedes aegypti*, *Chironomus tentans*, *Manduca sexta* and *Bombyx mori* (Figure 5). Interestingly, the N-terminal end of the *Heliothis* sequence has an in frame methionine start which is 20 amino acids longer than that reported for *Drosophila*, *Aedes aegypti* and *Manduca sexta*. However, the extended N-terminal end in the *Heliothis* EcR does not have similarity to that of *Bombyx mori*. Finally, the C-terminal end of the different B1 isoform 15 ecdysone receptor sequences diverge and do not have significant similarity.

C. Northern Blot Analysis

The sequence identified by screening the library is expected to be expressed in tissues undergoing developmental changes, thus mRNA from different developmental stages of *H. virescens* were isolated and a norther blot produced. The mRNAs were isolated from eggs; 1st, 2nd, 3rd, 4th and 5th instar larvae, pupae and adults. The northern blot was hybridised with a NdeI/XhoI DNA fragment from pSK19R encompassing the 3'end of the DNA binding domain through to the end of the ligand binding domain. The hybridisation was carried out in 1%(w/v)Marvel, 5X SSPE, 0.1%(w/v) SDS at 65°C for 18 to 24 hours. 25 The filters were washed in 3X SSPE + 0.1%(w/v) SDS and 1X SSPE + 0.1%(w/v) SDS at 65°C. The filter was blotted dry and exposed for one to seven days. The gene recognises two transcripts (6.0 and 6.5 kb) which appear to be expressed in all stages examined, however, the levels of expression differ in different stages. It should be noted that the same two transcripts are recognised by probes specific to the DNA binding domain and the ligand binding domain, 30 indicating that the two transcripts arise from the same gene either by alternative splicing or alternative use of polyadenylation sites.

In summary, adult and 5th instar larvae have lower levels of expression while all other tissues have substantial levels of expression.

Example II Expression of *Heliothis* ecdysone receptor in Mammalian cells

To demonstrate that the cDNA encodes a functional ecdysone receptor, effector constructs were generated containing the HEcR under the control of the CMV (cytomegalovirus) promoter, and the DNA expressed in mammalian cells.

Effector constructs

A first mammalian expression plasmid was constructed by placing a HindIII/NotI pSK19R fragment into the pcDNA3 HindIII/NotI vector resulting in pcDNA319R (Figure 7).

A second effector plasmid was constructed wherein the non-coding region of the cDNA 19R was deleted and a consensus Kozak sequence introduced. The mutagenesis was carried out by PCR amplifying a DNA fragment with the oligo HecRH3C :

5'aattaagcttccaccatgccgttaccaatgccaccgaca 3'

containing a unique HindIII restriction enzyme recognition site followed by the mammalian Kozak consensus sequence, and HecRNdeI :

5'cttcaaccgacactcctgac 3'.

The resulting 353bp PCR fragment was restriction enzyme digested with HindIII and NdeI, gel purified and ligated with 19R NdeI/NotI fragment into a pcDNA3 HindIII/NotI vector resulting in pcDNA3HecR.

A third effector construct was made with the 5' end sequences of pSK16.1 by PCR. The PCR approach involved PCR amplifying the 5' end sequences using a 5' oligonucleotide containing a HindIII restriction cloning site, the Kozak consensus sequence followed by nucleotide sequence encoding for a Methionine start and two Arginines to be added to the 5' end of the amplified fragment :

(16H3K 5' attaagcttgcgcgcatgcgcgcacgctggataacaacggaccattc 3'),
the 3' oligonucleotide used was HecrNdeI. The resulting fragment was restriction enzyme digested, gel purified and subcloned with an NdeI/NotI 19R fragment into pcDNA3 NdeI/NotI vector. The plasmid was named pcDNA3H3KHEcR.

A fourth effector construct was produced which contains the extended N-terminal end sequence obtained from the 5'RACE experiment. Thus, a PCR approach was followed to introduce the new 5' end sequences in addition to a consensus Kozak sequence and a HindIII unique cloning sequence. The sense oligonucleotide used was RACEH3K :

5' attaagcttgcgcgcatgtccctcgccgctcgat 3',

while the antisense primer was the same as that used before (HecrNdeI). The cloning strategy was the same as used for the pcDNA3H3KHEcR to give rise to pcDNA3RACEH3KHEcR.

The PCR mutagenesis reactions were carried out in the same manner for all constructs. The PCR conditions used were 1 minute at 94°C, 1 minute at 60°C and 1 minute

at 72°C for 15 cycles. The reactions conditions were 50mM Tris-HCl (pH8.4), 25mM KCl, 200mM dNTPs (dATP, dCTP, dGTP and dTTP), 200nM of each oligonucleotide and 2.5U/Reaction of *Taq* DNA polymerase. For each construct at least 5 independant PCR reactions were carried out and several clones were sequenced to insure that at least one is mutation free.

5 Reporter construct

The reporter plasmid to be co-transfected with the expression vector contained 4 copies of the Hsp27 ecdysone response element (Riddihough and Pelham, 1987) fused to B-globin promoter and the B-Galactosidase gene. The tandem repeats of the ecdysone response 10 element were synthesised as two complementary oligonucleotides which when annealed produced a double standed DNA molecule flanked by an SpeI site at the 5' end and a ClaI site at the 3' end :

Recr3A

15 5'ctagtagacaagggtcaatgcacttgtccaataagcttagacaagggtcaatgcacttgtccaatgaattcagacaagggtcaat gcacttgtccaaatctgcagagacaagggtcaatgcacttgtccatat 3'

Recr3B

5'cgatattggacaagtgcattgaacccttgcattgcagattggacaagtgcattgaacccttgcattgaaattcattggacaagtgcattg aacccttgcattgaaattgcattgaacccttgcatt 3'.

20 The resulting 135bp DNA fragment was ligated to the vector pSWBGAL SpeI/ClaI resulting in pSWREcR4 (Figure 8). The co-transfection of the two plasmid should result in B-galactosidase activity in the presence of ligand. The experiment relies upon the presence of RXR (a homologue of ultraspiracle) in mammalian cells for the formation of an active ecdysone receptor.

25 Mammalian transfection methods

Transfections of mammalian cell lines (CHO-K1 Chinese hamster ovary)- ATCC number CCL61 or cos-1 (Monkey cell line) were performed using either calcium phosphate precipitation (Gorman, Chapter 6 of "DNA cloning: a practical approach. Vol 2 D.M. Glover ed.(1985) IRL Press, Oxford) or using LipofectAMINE (Gibco BRL Cat. No. 18324-012, following manufacturers instructions). Human Epithelial Kidney 293 cells were transfected 30 using analogous methods.

Results - Native HEcR drives transient reporter gene expression in mammalian cells

Co-transfection of pcDNA3H3KHEcR (Effector) and reporter constructs into Human Epithelial Kidney 293 cells (HEK293) in the presence of either Muristerone A or RH5992 resulted in a 2-3 fold induction of reporter activity compared to the no chemical controls 35 (Figure 9). The HEK293 cells were used since they are known to have constitutive levels of α RXR which have been demonstrated to be necessary for *Drosophila* EcR activation by Muristerone A (Yao., et al., 1993). Moreover, to further investigate the need for RXR

interactions, a α RXR was co-transfected into HEK293 cells (along with the effector and reporter) resulting in a 9 fold induction of reporter activity compared to the untreated cells (Figure 9). The co-transfection of α RXR with reporter and effector increased by four fold the reporter activity compared to cells transfected with effector and reporter alone. Induction was
5 observed both in the presence of either Muristerone A or RH5992. These data clearly demonstrate that the cDNA HEcR encodes a functional ecdysone receptor. Moreover, The ability of HEcR to complex with α RXR and bind Muristerone A or RH5992 provide evidence for the usage of the entire HEcR as a component of a mammalian gene switch. In particular, it offers the advantage of reducing uninduced expression of target gene since ecdysone
10 receptor and response elements are not present in mammalian cells.

Example III - Chimeric constructs and ligand validation in Maize Protoplasts

In order to apply the ecdysone receptor as an inducible system it was deemed necessary
15 to simplify the requirements of the system by avoiding the need of a heterodimer formation to obtain an active complex. The glucocorticoid receptor is known to form homodimers and chimeric constructs of the glucocorticoid receptor transactivating and DNA binding domains fused to the ecdysone receptor hinge and ligand binding domains have been shown to be active as homodimers in mammalian cells in the presence of Muristerone A (an ecdysone
20 agonist)(Christopherson et al., 1992). However, the chimeric receptor is not responsive to 20-hydroxyecdysone (Christopherson et al., 1992).

The analysis of the activation of the glucocorticoid/*Heliothis* ecdysone chimeric receptor entailed the production of two other control effector constructs. The first one of the constructs contained the intact glucocorticoid receptor while the second one contained a
25 glucocorticoid/*Drosophila* ecdysone chimeric receptor.

Effector constructs

(i) Glucocorticoid receptor Maize expression construct

The glucocorticoid receptor DNA for the Maize transient expression construct was produced via the polymerase chain reaction (PCR) of Human Fibrosarcoma cDNA (HT1080
30 cell line, ATCC#CC1121) library (Clontech)(see Hollenberg et al., 1985). The PCR approach taken was to amplify the 2.7kb fragment encoding the glucocorticoid receptor in two segments. The first segment entails the N-terminal end up to and including the DNA binding domain while the second fragment begins with the hinge region (amino acid 500) thought to the end of the reading frame. Thus, the PCR primer for the N-terminal end segment was
35 designed to contain an EcoRI site and the Kozak consensus sequence for translation initiation
:

GREcoRI 5'attgaattccaccatggactccaaagaatcattaactc 3'.

The 3'end primer contains a XhoI site in frame with the reading frame at amino acid 500 of the published sequence :

GRXhoI 5' gagactccctgttagtggcctc gagcatttttttttttc 3'.

5 The second fragment of the glucocorticoid receptor was produced with a 5' end oligonucleotide containing an XhoI site in frame with the open reading frame at the begining of the hinge region (amino acid 500) :

GRHinge 5' atttccgagattcagcaggccactacaggag 3'

while the 3' end oligonucleotide contained an EcoRI site 400 bp after the stop codon :

GRStop 5' attgaattcaatgctatcgtaaactatacaggg 3'.

10 The glucocorticoid receptor PCR was carried out using Vent polymerase (Biolabs) under hot start conditions followed by 15 cycles of denaturing (94°C for 1 minute), annealing (66°C for 1 minute) and DNA synthesis (72°C for 3 minute). The template was produced by making first strand cDNA as described in the TA cloning kit (Invitrogen) after which the PCR was carried out in 10mM KCl, 10mM (NH₄)₂SO₄, 20mM TRIS-HCl pH 8.8, 2 mM MgSO₄, 15 0.1% (v/v) Triton X-100, 200 mM dNTPs, 100ng of each Primer and 2 U of Vent Polymerase. The PCR products was restriction enzyme digested with EcoRI and XhoI and subcloned into pBluescript SK (pSK) EcoRI. The resulting plasmid pSKHGI was sequenced and found to lack any mutations from the published sequences (apart from those introduced in the PCR primers) (Hollenberg et al., 1985).

20 The 2.7kb EcoRI fragment was subcloned into the vector pMF6PAT EcoRI resulting in pMF6HGIPAT (Figure 10).

(ii) Maize expression construct containing a Glucocorticoid/ *Drosophila* ecdysone chimeric receptor.

25 The glucocorticoid receptor portion of the chimeric receptor was isolated from pSKHGI by producing a 1.5kb BamHI/XhoI restriction fragment containing the N-terminal end up to and including the DNA binding domain.

30 The *Drosophila* ecdysone receptor portion was isolated through PCR of first stand cDNA prepared from *Drosophila* adult mRNA. The PCR was carried out using a 5' oligonucleotide containing a SalI site (ie. *Drosophila* ecdysone receptor contains a XhoI site at the end of the ligand binding domain) which starts at the begining of the hinge region : amino acid 330, Ecr8 attgtcgacaacggccggaatggctcgccccggag 3'.

The 3' end oligonucleotide contains an BamHI site adjacent to the stop codon : EcRstop 5' tcgggcttttaggatcctaagccgtggcgaatgcctccgacttaac 3'.

35 The PCR was carried out under the conditions described for the amplification of the Glucocorticoid receptor and yielded a 1.6 kb fragment. The fragment was introduced into

pSK SalI/BamHI and the sequence determined and compared to the published one (Koelle et al., 1991).

The maize transient expression plasmid was produced by introducing into pMF6 BamHI vector the 1.5kb BamHI/XhoI glucocorticoid receptor fragment and the 1.6kb 5 SalI/BamHI *Drosophila* receptor portion to yield the chimeric plasmid pMF6GREcRS (Figure 9).

(iii) Construction of the Glucocorticoid/*Heliothis* ecdysone chimeric receptor Maize transient expression plasmid.

10 The Glucocorticoid receptor portion of the chimera was produced as describe in Example II(ii). The production of the *Heliothis* ecdysone receptor portion involves the introduction of a SalI recognition site at the DNA binding/hinge domain junction (amino acid 229). The addition of the SalI site :

Hecrsal 5'attgtcgacaaaggcccgagtgcgtggtgccggag 3'

15 was achieved via PCR mutagenesis making use of an unique AccI site 107bp downstream of the juction point (or 1007 bp relative to Seq 1D No 4):

Hecracc 5' tcacattgcatgatggaggcatg 3'.

20 The PCR was carried out using *Taq* polymerase (2.5 U) in a reaction buffer containing 100ng of template DNA (pSK19R), 100ng of Hecrsal and Hecracc, 20 mM TRIS-HCl pH 8.4, 50mM KCl, 10mM MgCl₂, 200mM dNTPs. The reaction was carried out with an initial denaturation of 3 minutes followed by 15 cycles of denaturation (1 minute at 94°C), annealing (1 minute at 60°C) and DNA synthesis (1 minute at 72°C). The DNA was restriction enzyme digested and subcloned into pSK SalI/SacI with the 1.2kb AccI/SacI 3' end HecR fragment to yield pSK HeCRDEF (or containing the hinge and ligand binding domains of the *Heliothis* 25 ecdysone receptor). The construction of the maize transient expression plasmid containing the Glucocorticoid/*Heliothis* ecdysone chimeric receptor involved the ligation of pMF6 EcoRI/SacI with the 1.5kb EcoRI/XhoI fragment of Glucocorticoid receptor N-terminal end and the 1.2 kb SalI/SacI fragment of pSk HEcRDEF to yield pMF6GRHEcR (Figure 10).

Reporter plasmids

30 Two reporter plasmids were made by inserting the into p221.9 or p221.10 BamHI/HindIII vectors two pairs or oligonucleotides containing six copies of the glucocorticoid response element (GRE). The two sets of oligonucleotides were designed with restriction enzyme recognition sites so as to ensure insertion of the two pairs in the right orientation. The first oligonucleotide pair GRE1A/B is 82 nucleotides long and when annealed 35 result in a DNA fragment flanked with a HindIII site at the 5' end and a SalI site at the 3' end : GRE1A

5'agcttcgactgtacaggatgttctagctactcgagtagctagaacatcctgtacagtgcagtagctagaacatcctgtacag 3'

GRE1B

5'tcgactgtacaggatgtctagctactcgactgtacaggatgtctagctactcgagtcgctagaacatccgtac agtcga 3'.

The second pair of oligonucleotides is flanked by a SalI site at the 5' end and a BamHI site at the the 3' end

5 GRE2A 5' tcgacttagctagaacatccgtacagtgcgatgtactcgactgtacaggatgtctagctactcgactgtac agtcgatgtactcgactgtacacatccgtacag 3'

GRE2B 5'gatccgtacaggatgtctagctactcgactgtacaggatgtctagctactcgactgtacaggatgtctagctac tag 3'.

The resulting plasmids were named p221.9GRE6 (Figure 13) and p221.10GRE6 (Figure 14)(used in later Example). The difference between p221.9 and p221.10 plasmids is 10 that p221.9 contains the -60 35SCaMV minimal promotor while p221.10 (p221.10GRE6) contains the -46 35SCaMV minimal promotor.

Method

Protoplasts were isolated from a maize suspension culture derived from BE70 x A188 embryogenic callus material, which was maintained by subculturing twice weekly in MS0.5_{mod}. 15 (MS medium supplemented with 3% sucrose, 690mg/l proline, 1g/l myo-inositol, 0.2g/l casein acid hydrolysate, 0.5mg/l 2,4-D, pH5.6). Cells from suspensions two days post subculture were digested in enzyme mixture (2.0% Cellulase RS, 0.2% Pectolyase Y23, 0.5M Mannitol, 5mM CaCl₂H₂O, 0.5% MES, pH5.6, ~660mmol/kg) using ~10ml/g cells, incubating at 25°C, dim light, rotating gently for ~2 hours. The digestion mixture was sieved sequentially through 20 250µm and 38µm sieves, and the filtrate centrifuged at 700rpm for 3.5 minutes, discarding the supernatant. The protoplasts were resuspended in wash buffer (0.358M KCl, 1.0mM NH₄NO₃, 5.0mM CaCl₂H₂O, 0.5mM KH₂PO₄, pH4.8, ~670mmol/kg) and pelleted as before. This washing step was repeated. The pellet was resuspended in wash buffer and the protoplasts were counted. Transformation was achieved using a Polyethylene glycol method 25 based on Negruiti et.al. Protoplasts were resuspended at 2 x 10⁶/ml in MaMg medium (0.4M Mannitol, 15mM MgCl₂, 0.1% MES, pH5.6, ~450mmol/kg) aliquotting 0.5ml / treatment (i.e. 1x10⁶ protoplasts / treatment). Samples were heat shocked at 45°C for 5 minutes then cooled to room temperature. 10µg each of p221.9GRE6 and pMF6HR1PAT (GR) (1mg/ml) / treatment were added and mixed in gently, followed by immediate addition of 0.5ml warm (~45°C) PEG solution (40% PEG 3,350MW in 0.4M Mannitol, 0.1M Ca(NO₃)₂, pH8.0), 30 which was mixed in thoroughly but gently. Treatments were incubated at room temperature for 20-25 minutes, then 5ml 0.292M KCl (pH5.6, ~530mmol/kg) was added step-wise, 1ml at a time, with mixing. The treatments were incubated for a further 10-15 minutes prior to pelleting the protoplasts by centrifuging as before. Each protoplast treatment was 35 resuspended in 1.5ml culture medium (MS medium, 2% sucrose, 2mg/l 2,4-D, 9% Mannitol, pH5.6, ~700mmol/kg) +/- 0.0001M dexamethasone (glucocorticoid). The samples were incubated in 3cm dishes at 25°C, dark, for 24-48 hours prior to harvesting. Fluorometric

assays for GUS activity were performed with the substrate 4-methylumbelliferyl-D-glucuronide using a Perkin-Elmer LS-35 fluorometer (Jefferson et al., 1987). Protein concentration of tissue homogenates were determined by the Bio-Rad protein assay (Bradford, 1976). The method was repeated for each effector construct.

5 Results

Reporter gene assay

A reporter gene construct (p221.9GRE6) was generated containing the GUS reporter gene under the control of a -60 CaMV 35S promoter with 6 copies of the glucocorticoid response element. To test this construct was functional in maize protoplasts a co-transformation assay was performed with the reporter construct p221.9GRE6 and the effector construct pMF6HR1PAT (GR) construct containing the entire glucocorticoid receptor.

Figure 15 shows that Reporter p221.9GRE6 alone or reporter plus effector pMF6HR1PAT (GR) with no activating chemical gave no significant expression. When reporter plus effector were co-transformed into maize protoplasts in the presence of 0.0001M dexamethasone (glucocorticoid), a significant elevation of marker gene activity was observed (Figure 15). The response is specific to glucocorticoid as the steroid Muristerone A does not lead to induced levels of expression. These studies clearly show the reporter gene construct p221.9GRE6 is capable of monitoring effector /ligand mediated gene expression.

15 Chimeric ecdysone effector constructs mediate inducible expression in maize transient

20 protoplasts assays

A chimeric effector plasmid pMF6GREcRS was constructed, containing the ligand binding domain from the *Drosophila* ecdysone receptor and the DNA binding and transactivation domain from the glucocorticoid receptor. To confirm the reporter gene construct p221.9GRE6 could respond to a chimeric ecdysone effector construct, a series of co-transformation into maize protoplasts was performed.

Figure 16 shows that reporter (p221.9GRE6) alone or reporter plus effector (pMF6GREcRS) with no activating chemical, gave no significant expression in maize protoplasts. When reporter plus effector were co-transformed into maize protoplasts in the presence of 100 μ M Muristerone A, a significant elevation of marker gene activity was observed. The response was specific to Muristerone A, as the steroid dexamethasone did not lead to induced levels of expression. These studies clearly showed the reporter gene construct p221.9GRE6 is capable of monitoring chimeric ecdysone effector /ligand mediated gene expression.

30 A second chimeric effector construct pMF6GRHEcR, was generated containing the ligand binding domain from *Heliothis* ecdysone receptor. When co-transformed into maize protoplasts with the reporter plasmid p221.9GRE6, no response to 100 μ M Muristerone or

100 μ M dexamethasone was observed (Figure 17). These data clearly show the *Drosophila* and *Heliothis* ligand binding domains exhibit different properties.

When the effector plasmid pMF6GREcRS, containing the ligand binding domain from 5 *Drosophila*, was tested with the reporter p221.9GRE6 in presence of the non-steroidal ecdysone agonists RH5849 and RH5992 (mimic), no chemical induced reporter gene activity was observed (Figures 18 and 19).

When the effector plasmid pMF6GRHEcR, containing the ligand binding domain from 10 *Heliothis*, was tested with the reporter p221.9GRE6 in presence of the non-steroidal ecdysone agonists RH5992 (mimic), significant chemical induced reporter gene activity was observed (Figure 20). These data demonstrate the ligand binding domain from *Heliothis* has different properties to the *Drosophila* receptor in that the former responded to the non-steroidalecdysteroid agonist RH5992. Figure 21 demonstrates the effector plasmid pMF6GRHEcR confers RH5992 dependant inducibility on the reporter p221.9GRE6 in a dose responsive manner. Induction was observed in a range from 1 μ M-100 μ M RH5992.

15

Example IV - Testing of effector vectors in Tobacco protoplasts

The experiments carried out in the previous example demonstrated the specific effect 20 of RH5992 (mimic) on pMF6GRHEcR in maize protoplasts. It is the aim in this example to show the generic application to plants of the glucocorticoid/*Heliothis* ecdysone chimeric receptor switch system. Tobacco shoot cultures cv. Samsun, were maintained on solidified MS medium + 3% sucrose in a controlled environment room (16 hour day / 8 hour night at 25°C, 55% R.H), were used as the source material for protoplasts. Leaves were sliced parallel to the mid-rib, discarding any large veins and the slices were placed in CPW13M 13% 25 Mannitol, pH5.6, ~860mmol/kg) for ~1 hour to pre-plasmolysate the cells. This solution was replaced with enzyme mixture (0.2% Cellulase R10, 0.05% Macerozyme R10 in CPW9M (CPW13M but 9% Mannitol), pH5.6, ~600mmol/kg) and incubated in the dark at 25°C overnight (~16 hours). Following digestion, the tissue was teased apart with forceps and any large undigested pieces were discarded. The enzyme mixture was passed through a 75 μ m 30 sieve and the filtrate was centrifuged at 600rpm for 3.5 minutes, discarding the supernatant. The pellet was resuspended in 0.6M sucrose solution and centrifuged at 600rpm for 10 minutes. The floating layer of protoplasts was removed using a pasteur pipette and diluted with CPW9M (pH5.6, ~560mmol/kg). The protoplasts were again pelleted by centrifuging at 600rpm for 3.5 minutes, resuspended in CPW9M and counted. A modified version of the 35 PEG-mediated transformation above was carried out. Protoplasts were resuspended at 2x10⁶/ml in MaMg medium and aliquotted using 200 μ l / treatment (i.e. 4x10⁵ protoplasts / treatment). 20 μ g each of pMF6GRHEcRS and p221.9GRE6 DNA (1mg/ml) were added

followed by 200 μ l PEG solution and the solutions gently mixed. The protoplasts were left to incubate at room temperature for 10 minutes before addition of 5ml MSP19M medium (MS medium, 3% sucrose, 9% Mannitol, 2mg/l NAA, 0.5mg/l BAP, pH5.6, ~700mmol/kg) +/- 10 μ M RH5992. Following gentle mixing, the protoplasts were cultured in their tubes, lying horizontally at 25°C, light. The protoplasts were harvested for the GUS assay after ~24 hours.

5 Effector construct

(i) Construction of a Dicotyledonous expression vector

The vector produced is a derivative of pMF6. pMF6GREcRS was restriction enzyme digested with PstI to produce 3 fragments namely, 3.4(Adh Intronless pMF6), 3.2(GREcRS) 10 and 0.5(Adh intron I) kb). Isolation and religation of the 3.4 and 3.2 kb fragments resulted in pMF7GREcRS (Figure 22). pMF7GREcRS was restriction enzyme digested with EcoRI/SacI resulting in the 3.4kb pMF7 EcoRI/SacI vector which when isolated and purified was ligated to a 1.5 kb EcoRI/XbaI N-terminal end of the glucocorticoid receptor and the 1.2 kb 15 SalI/SacI *Heliothis* ecdysone C-terminal end sequences to produce pMF7GRHEcR (Figure 23).

Reporter plasmid

The reporter plasmids constructed for the maize transient experiments were the same as those used without alteration in the tobacco leaf protoplast transient expression experiments.

20 Results - Chimeric ecdysone effector constructs mediate inducible expression in tobacco transient protoplast assays

Experiments were performed to demonstrate that the effector plasmid pMF7GRHEcR can confer chemical dependant inducible expression on the reporter p221.9GRE6 in tobacco mesophyll protoplasts.

25 Figure 24 shows that reporter (p221.9GRE6) alone or reporter plus effector (pMF7GRHEcR) with no activating chemical, gave no significant expression in tobacco protoplasts. When reporter plus effector were co-transformed into tobacco protoplasts in the presence of 10 μ M RH5992, a significant elevation of marker gene activity was observed. These data show a chimeric ecdysone effector construct, containing the *Heliothis* ligand 30 binding domain can confer non-steroidal ecdysteroid dependant expression on reporter gene constructs in both monocotyledonous and dicotyledonous species.

Example V - Chimeric activity in Mammalian cells

Effector constructs

5 (i) Construction of Glucocorticoid/*Heliothis* ecdysone chimeric receptor.

The mammalian expression vector used in this experiment was pcDNA3 (Invitrogen). The GRHEcR 2.7kb BamHI DNA fragment (isolated from pMF6GRHEcR) was introduced into the pcDNA3 BamHI vector. The recombinants were oriented by restriction enzyme mapping. The DNA sequence of the junctions was determined to ensure correct orientation 10 and insertion (pcDNA3GRHEcR, Figure 25).

Reporter construct

The reporter plasmid for mammalian cell system was produced by taking pSWBGAL plasmid and replacing the CRESW SpeI/Clal fragment for a synthetic 105 bp DNA fragment containing 4 copies of the glucocorticoid response element (GRE) and flanked by SpeI at the 15 5' end and AflII at the 3' end.

The oligonucleotides were synthesised using the sequences :

GREspeI

5'ctagttgtacaggatgttctagctactcgagtagctagaacatcctgtacagtgcagtagctagaacatcctgtacagtgcagtagct
agaacatcctgtacac 3'

20 GREafl2

5'ttaagtgtacaggatgttctagctactcgactgtacaggatgttctagctactcgactgtacaggatgttctagctactcgagtagcta
gaacatcctgtacaa 3'.

The two oligonucleotides were purified annealed and ligated to pSWBGAL SpeI/AflII to produce pSWGRE4 (Figure 26).

25 Results - Chimeric HEcR drives transient reporter gene expression in mammalian cells

No expression was detected when a reporter gene construct pSWGRE4, comprising of a minimal β-globin promoter containing four copies of the glucocorticoid response element, fused to a β-galactosidase reporter gene, was introduced into CHO cells. Similarly, no expression was detected when pSWGRE4 and an effector plasmid pCDNA3GRHEcR, 30 containing the transactivation and DNA binding domain from the glucocorticoid receptor and the ligand binding domain from the *Heliothis* ecdysone receptor, under the control of the CMV promoter were co-transformed into CHO-K1 or HEK293 cells. When co-transformed CHO (Figure 27) and HEK293 cells (Figure 28) were incubated in the presence of the non-steroidal ecdysone agonists RH5992 (mimic), significant chemical induced reporter gene 35 activity was observed. Equally, induction of reporter activity was observed when HEK293 cells transfected with pcDNA3GRHEcR and reporter were treated with Muristerone A (Figure 28).

Example VI - Screening system allows new chemical activators and modified ligand binding domains to be tested in Mammalian cells

5 The basis of a screening system are in place after the demonstration that the chimeric receptor was activated in the presence of RH5992. A screen was carried out using CHO cells transiently transfected with both pSWGREG4 (reporter) and pcDNA3GRHEcR (effector) constructs. In the first instance 20 derivatives compounds of RH5992 were screened. It was observed that 7 out of the 20 compounds gave an increased reporter gene activity compared
10 to untreated cells. A second screen was carried out in which 1000 randomly selected compounds were applied to transiently transfected CHO cells. Two compounds were found to activate reporter gene activity above that from the untreated controls. The second screen suggest that this cell based assay is a robust and rapid way to screen a small library of compounds, where a thousand compounds can be put through per week.

15

Example V - Stably transformed Tobacco plants

Stable Tobacco vectors

20 The components of the stable Tobacco vectors were put together in pBluescript prior to transfer into the binary vector. The production of stable transformed plants entails the production of a vector in which both components of the switch system (ie. effector and reporter) are placed in the same construct to then introduce into plants.

25 The methodology described below was used to produce four different stable Tobacco vectors. The method involves three steps:

25

1. pBluescript SK HindIII/EcoRI vector was ligated to either GRE6-4635SCaMVGUSNOS HindIII/EcoRI (from p221.10GRE6) or GRE6-6035SCaMVGUSNOS HindIII/EcoRI (from p221.9GRE6) resulting in plasmid pSK-46 and pSK-60.

30

2. This step involves the addition of the chimeric receptor (35SGRHEcRNOS or 35SGRVP16HEcRNOS) to pSK-60 or pSK-46. Thus a pSK-60 (or pSK-46) XbaI vector was ligated with either the 3.4kb 35SGRHEcRNOS XbaI or the 3.0kb 35SGRVP16HEcRNOS XbaI DNA fragment to produce pSKES1 (pSKGRE6-6035SCaMVGUSNOS-35SGRHEcRNOS), pSKES2 (pSKGRE6-4635SCaMVGUSNOS-35SGRHEcRNOS), pSKES3 (pSKGRE6-6035SCaMVGUSNOS-35SGRVP16HEcRNOS) and pSKES4 (pSKGRE6-4635SCaMVGUSNOS-35SGRVP16HEcRNOS).

3. Transfer from pBluescript based vectors to binary vectors. The transfer of the ES1 (Figure 29) ES2 (Figure 30), ES3 (Figure 31) or ES4 (Figure 32) DNA fragments into the binary vector JRI involves five steps:

5

(i) Restriction enzyme digestion of pSKES1 (ES2, ES3, and ES4) with ApaI and NotI to liberate the insert from the vector pBluescript.

(ii) The two DNA fragments were BamHI methylated for 2 hours at 37°C in TRIS-HCl, MgCl₂, 80uM SAM (S-adenosylmethionine) and 20 U of BamHI methylase.

10 (iii) Ligate a ApaI/NotI linker onto the fragment. The linker was designed to have an internal BamHI site :

ApaBNot1 5' cattggatcccttagc 3' and

ApaBNot2 5'ggccgcataaggatccaatgggcc 3'.

(iv) Restriction enzyme digest the protected and linked fragment with BamHI and

15 fractionate the products on a 1%(w/v) Agarose gel. The protected DNA fragment (5.5kb) was cut out of the gel and purified.

(v) A ligation of JRI BamHI vector with the protected band was carried out to produce JRIES1 (JRIES2, JRIES3 or JRIES4). The DNA of the recombinant was characterised by restriction mapping and the sequence of the junctions determined.

20 The plant transformation construct pES1, containing a chimeric ecdysone receptor and a reporter gene cassette, was transferred into Agrobacterium tumefaciens LBA4404 using the freeze/thaw method described by Holsters et al. (1978). Tobacco (*Nicotiana tabacum* cv Samsun) transformants were produced by the leaf disc method (Bevan, 1984). Shoots were regenerated on medium containing 100mg/l kanamycin. After rooting, plantlets were transferred to the glasshouse and grown under 16 hour light/ 8 hour dark conditions.

25 Results - Chimeric ecdysone effector constructs mediate inducible expression in stably tobacco plants

Transgenic tobacco plants were treated in cell culture by adding 100μM RH5992 to MS media. In addition seedlings were grown hydroponically in the presence or absence of RH5992. In further experiments 5mM RH5992 was applied in a foliar application to 8 week old glasshouse grown tobacco plants. In the three methods described uninduced levels of GUS activity were comparable to a wild type control, while RH5992 levels were significantly elevated.

Ecdysone switch modulation and optimisation**Example VIII - Yeast indicator strains for primary screen of chemical libraries**

5

A set of yeast indicator strains was produced to use as a primary screen to find chemicals which may be used in the gene switch. The properties of the desired chemicals should include high affinity resulting in high activation but with different physico-chemical characteristics so as to increase the scope of application of the technology. Moreover, the 10 production of this strain also demonstrates the generic features of this switch system.

Effector vector

A base vector for yeast YCp15Gal-TEV-112 was generated containing:

Backbone - a modified version of pRS315 (Sikorski and Hieter (1989) Genetics 122, 19-27)- a shuttle vector with the LEU2 selectable marker for use in yeast;

15 ADH1 promoter (BamHI- Hind III fragment) and ADH1 terminator (Not I- Bam HI fragment) from pADNS (Colicelli et al PNAS 86, 3599-3603);

DNA binding domain of GAL4 (amino acids 1-147; GAL4 sequence is Laughon and Gesteland 91984) Mol. Cell Biol. 4, 260-267) from pSG424 (Sadowski and Ptashne (1989) Nuc. Acids Res. 17, 7539);

20 Activation domain - an acidic activation region corresponding to amino acids 1-107 of activation region B112 obtained from plasmid pB112 (Ruden et al (1991) Nature 350, 250-252).

The plasmid contains unique Eco RI, Nco I and Xba I sites between the DNA binding domain and activation domains.

25 Into this vector a PCR DNA fragment of the *Heliothis* ecdysone receptor containing the hinge, ligand binding domains and the C-terminal end was inserted. The 5' oligonucleotide is flanked by an NcoI restriction recognition site and begins at amino acid 259 :

HecrNcoI 5' aattccatggtagacgacagtagacatcac 3'.

The 3' oligonucleotide is flanked by an XbaI site and encodes for up to amino acid 30 571:

HecRXbaI 5' ctgaggcttagagacgggtggcggcggcc 3'.

The PCR was carried out using vent polymerase with the conditions described in Example IA. The fragment was restriction enzyme digested with NcoI and XbaI purified and ligated into YCp15GALTEV112 NcoI/XbaI vector to produce YGALHeCRB112 or TEV-35 B112 (Figure 34). In order to reduce constitutive activity of the YGALHeCRB112 plasmid a YGALHeCR plasmid was produced in which the B112 activator was deleted by restriction enzyme digesting YGALHeCRB112 with XbaI/SpeI followed by ligation of the resulting

vector (ie. SpeI and XbaI sites when digested produce compatible ends)(TEV-8, Figure 33). An effector plasmid was constructed whereby the B112 transactivating domain was excised from YGalHecRB112 with XbaI and replaced with the VP16 transactivation domain DNA fragment (encoding amino acids 411 and 490 including the stop codon). The resulting vector
5 was named YGalHecRVP16 or TEVVP16-3 (Figure 35).

Reporter construction for yeast

The *S. cerevisiae* strain GGY1::171 (Gill and Ptashne (1987) Cell 51, 121-126), YT6::171 (Himmelfarb et al (1990) Cell 63, 1299-1309) both contain reporter plasmids consisting of the GAL4-responsive GAL1 promoter driving the *E. coli* B-galactosidase gene.
10 These plasmids are integrated at the URA3 locus. The reporter strain YT6::185 contains the reporter plasmid pJP185 (two synthetic GAL4 sites driving the B-galactosidase gene) integrated at the URA3 locus of YT6 (Himmelfarb et al). (Note- the parental strains YT6 and GGY1 have mutations in the GAL4 and GAL80 genes, so the reporter genes are inactive in the absence of any plasmids expressing GAL4 fusions).

15 Yeast assay

Standard transformation protocols (Lithium acetate procedure) and selection of colonies by growth of cells on selective media (leucine minus medium in the case of the YCp15Gal-TEV-112 plasmid)- as described in Guthrie and Fink (1991) Guide to Yeast Genetics and Molecular Biology: Methods in Enzymology Vol. 194 Academic Press) and the reporter gene assay is a modification of that described in Ausabel et al (1993) Current Protocols in Molecular Biology (Wiley) Chapter 13.

Results - Automated screening system allows new chemical activators and modified ligand binding domains to be tested in yeast

An effector vector pYGALHEcRB112 has been generated containing a GAL4 DNA binding domain, a B112 activation domain and the ligand binding region from *Heliothis virescens*. In combination with a GAL reporter vector, pYGALHEcRB112 form the basis of a rapid, high throughput assay which is cheap to run. This cell-based assay in yeast (*Saccharomyces cerevisiae*) will be used to screen for novel non-steroidal ecdysone agonists which may of commercial interest as novel insecticides or potent activators of the ecdysone
30 gene switch system. The demonstration of an efficient system to control gene expression in a chemical dependant manner, forms the basis of an inducible system for peptide production in yeast.

The yeast screening system forms the basis of a screen for enhanced ligand binding using the lac Z reporter gene vector to quantitatively assay the contribution of mutation in the ligand binding domain. Alternatively, enhanced ligand binding capabilities or with a selection cassette where the lac Z reporter is replaced with a selectable marker such as uracil (URA 3), tryptophan (Trp1) or leucine (Leu2), and histidine (His). Constructs based on

pYGALHEcRB112 with alterations in the ligand binding domain are grown under selection conditions which impair growth of yeast containing the wild type ligand binding domain. Those surviving in the presence of inducer are retested and then sequenced to identify the mutation conferring resistance.

5

Example IX - Optimisation of chimeric receptor using a strong transactivator

Construction of mammalian expression plasmid with chimeric receptor containing Herpex Simplex VP16 protein sequences.

10 The construction of this chimeric receptor is based on replacing the sequences encoding for the glucocorticoid receptor transactivating domain with those belonging to the VP16 protein of Herpex simplex. Thus PCR was used to generate three fragments all to be assembled to produce the chimeric receptor. The PCRs were carried out as described in Example II, iii. The first fragment includes the Kozak sequences and methionine start site of 15 the glucocorticoid receptor to amino acid 152 of the glucocorticoid receptor. The oligonucleotides used for the generation of this fragment included an EcoRI site at the 5' end: GR1A 5' atatgaattccaccatggactccaaagaatc 3'
and at the 3' end a NheI restriction enzyme recognition site :
GR1B 5' atatgctagctgtggggcagcacacagcagtgg 3'.

20 The second fragment also belongs to the glucocorticoid receptor and begins with a NheI site in frame with amino acid 406 :

GR2A 5'atatgctagctccagctcctcaacagcaacaac 3'

and ends with a XhoI site at amino acid 500 :

GR2B 5'atatctcgagcaattccctttattttttc 3'.

25 The two fragments were introduced into pSKEcoRI/SacI in a ligation containing GR1A/B EcoRI/NheI, GR2A/B NheI/XhoI and HEcR SalI/SacI (from pSKHEcRDEF) to yield pSKGRDHEcR. The GR sequences and junctions of the ligation were found to be mutation free.

30 The third fragment to be amplified was a sequence between amino acid 411 to 490 of the herpes simplex VP16 protein. The amplified fragment was flanked with SpeI recognition sites. SpeI produces compatible ends to those of NheI sites. The oligonucleotides used :

VP16C 5' attactagttctgcggcccccccgaccgat 3' and

VP16E 5' aattactagttccaccgtactcgtaattcc 3'

35 produced a 180 bp fragment which was restriction enzyme digested with SpeI and introduced into pSKGRΔHEcR NheI vector to produce pSKGRVP16HEcR. The DNA from the latter was sequenced and found to be mutation free, the junctions were also shown to be in frame with those of the glucocorticoid receptor.

The 2.2 kb EcoRV/NotI GRVP16HEcR fragment was introduced into a pcDNA3 EcoRV/NotI vector resulting in pcDNA3GRVP16HEcR (Figure 36).

Construction of plant transient expression effector plasmids containing the chimeric receptor with VP16 sequences

5 The same procedure was carried out to clone the GRVP16HeCR DNA fragment into tobacco(pMF7b) and maize(pMF6) expression vectors. A 2.2kb BarnHI DNA fragment was isolated from pcDNA3GRVP16HeCR and ligated in to the pMF6 BamHI (or pMF7b BamHI) vector to produce pMF6GRVP16HeCR (Figure 37) (or pMF7GRVP16HeCR) (Figure 38).

Results - Addition of strong activation domains enhance ecdysone switch system

10 The VP16 transactivation domain from herpes simplex virus has been added to a maize protoplast vector pMF6GRHEcR to generate the vector pMF6GRVP16HEcR. When co-transformed into maize protoplasts with the reporter construct p221.9GRE6, in the presence of 100µM RH5992, enhanced levels of expression were seen over pMF6GRHEcR. Figure 39, shows that RH5992 is able to induce GUS levels comparable to those observed 15 with the positive control (p35SCaMVGUS), moreover, a dose response effect is observable.

VP16 enhanced vectors (pES3 and pES4) have been generated for stable transformation of tobacco. Following transformation transgenic progeny containing pES3 and pES4, gave elevated GUS levels following treatment with RH5992, relative to comparable transgenic plants containing the non-VP16 enhanced vectors pES1 and pES2.

20 An enhanced mammalian vector pcDNA3GRVP16HEcR was prepared for transient transfection of mammalian cell lines. Elevated reporter gene activities were obtained relative to the effector construct (pcDNA3GRHEcR) without the VP16 addition.

"Acidic" activation domains are apparently "universal" activators in eukaryotes (Ptashne (1988) Nature 335 683-689). Other suitable acidic activation domains which have 25 been used in fusions are the activator regions of GAL4 itself (region I and region II; Ma and Ptashne (Cell (1987) 48, 847-853), the yeast activator GCN4 (Hope and Struhl (1986) Cell 46, 885-894) and the herpes simplex virus VP16 protein (Triezenberg et al (1988) Genes Dev. 2, 718-729 and 730-742).

30 Other acidic and non-acidic transcriptional enhancer sequences for example from plant fungal and mammalian species can be added to the chimeric ecdysone receptor to enhance induced levels of gene expression.

Chimeric or synthetic activation domains can be generated to enhance induced levels of gene expression.

Example X - Optimisation by replacement of *Heliothis* ligand binding domain in chimeric effector for that of an ecdysone ligand binding domain of another species

5 Mutagenesis of the ecdysone ligand binding domain results in the increased sensitivity
of the chimeric receptor for activating chemical. This can be achieved by deletions in the
ligand binding domain, use of error prone PCR (Caldwell et al., PCR Meth. Applic 2, 28-33
1992), and in vitro DNA shuffling PCR (Stemmer, Nature 370, 389-391 1994). To enhance
the efficacy of the listed techniques we have developed a screening system for enhanced levels
10 of induced expression (see below).

An alternative strategy to the mutation of a known ligand binding domain is to identify insect species which are particularly sensitive to ecdysteroid agonists. For example *Spodoptera exigua* is particularly sensitive to RH 5992. To investigate the role of the ecdysone receptor ligand binding domain in increased sensitivity to RH5992 we have isolated corresponding DNA sequences from of *S. exigua* (Figure 40, Sequence ID No. 6). Figure 41, Sequence ID No. 7 shows a protein alignment of the hinge and ligand binding domains of the *Heliothis virescens* and *Spodoptera exigua* ecdysone receptors. The protein sequence between the two species is well conserved.

Results - Manipulation of the ligand binding domain leads to enhanced induced expression

20 Isolation of an ecdysone ligand binding domain from another lepidopteran species was
carried out by using degenerate oligonucleotides and PCR of first strand cDNA (Perkin
Elmer, cDNA synthesis Kit) of the chosen species. The degenerate oligonucleotides at the 5'
end were HingxhoA and B and at the 3' end ligandxA/B

30 LigandxA 5' ttactcgagiacgtccaiatcttciaggaa 3'
 a t c a

ligandxB 5' ttactcgagiacgtcccaiatctcctciaagaa 3'
 a t t a

35

RNA was extracted from 4th instar larvae of *Spodoptera exigua* since *Spodoptera exigua* appears to be more sensitive to RH5992 than *Heliothis* (Smagghe and Degheele,

1994). The first strand cDNA was used in PCR reactions under the following conditions
20mM Tris-HCL (pH8.4), 50mM KCl, 1.5mM MgCl₂, 200mM dNTPs (dATP,dCTP,dGTP
and dTTP) and 0.02 U/ml *Taq* DNA polymerase and in the presence of 1ug of each Hinge (5'
3') and Ligand (5'3') oligonucleotides. The PCR cycling conditions were 94°C for 1 minute,
5 60°C for 2 minutes and 72°C for 1 minute and 35 cycles were carried out. A sample of the
completed reaction was fractionated in a 1% agarose (w/v) 1 x TBE gel, and the resulting 900
bp fragment was subcloned into pCRII vector (Invitrogen). The resulting clone (pSKSEcR 1-
10) were further characterised and sequenced.

10 **Example X - Manipulation of reporter gene promoter regions can modulate chemical
induced expression**

The context of the effector response element in the reporter gene promoter can be
used to modulate the basal and induced levels of gene expression. Six copies of the
15 glucocorticoid response element were fused to 46 bp or 60 bp of the CaMV 35S promoter
sequence. When used with the effector construct pMF7GRHEcRS the reporter gene
construct containing 46 bp of the CaMV 35S promoter gave reduced basal and induced levels
of GUS expression relative to the 60 bp reporter construct (Figure 42).

Constructs for plant transformation (pES1 and ES2) have been generated to
20 demonstrate the size of minimal promoter can be used to modulate the basal and induced
levels of gene expression in plants.

The number and spacing of response elements in the reporter gene promoter can be
adjusted to enhance induced levels of trans-gene expression.

The utility of a two component system (effector and reporter gene cassettes) allows
25 the spatial control of induced expression. Trans-gene expression can be regulated in an tissue
specific, organ specific or developmentally controlled manner. This can be achieved by
driving the effector construct from a spatially or temporally regulated promoter.

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- 40 -

SEQUENCE LISTING

(1) GENERAL INFORMATION:

5

(i) APPLICANT:

- (A) NAME: ZENECA LIMITED
- (B) STREET: 15 STANHOPE GATE
- (C) CITY: LONDON
- 10 (E) COUNTRY: UK
- (F) POSTAL CODE (ZIP): W1Y 6LN

(ii) TITLE OF INVENTION: A GENE SWITCH

15

(iii) NUMBER OF SEQUENCES: 7

(iv) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Floppy disk
- (B) COMPUTER: IBM PC compatible
- 20 (C) OPERATING SYSTEM: PC-DOS/MS-DOS
- (D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(vi) PRIOR APPLICATION DATA:

25

- (A) APPLICATION NUMBER: GB 9510759.5
- (B) FILING DATE: 26-MAY-1995

(vi) PRIOR APPLICATION DATA:

30

- (A) APPLICATION NUMBER: GB 9513882.3
- (B) FILING DATE: 07-JUL-1995

(vi) PRIOR APPLICATION DATA:

35

- (A) APPLICATION NUMBER: GB 9517316.7
- (B) FILING DATE: 24-AUG-1995

(vi) PRIOR APPLICATION DATA:

40

- (A) APPLICATION NUMBER: GB 9605656.9
- (B) FILING DATE: 18-MAR-1996

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

45

- (A) LENGTH: 116 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

50

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: Heliothis virescens

55 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

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60

AAATTCCGCC ATGCTTGGCA AATGGATATG TATATGCGGA GAAAATGCCA AGAGTA

116

60

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

- 41 -

(A) LENGTH: 1934 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: double
 (D) TOPOLOGY: circular

5

(ii) MOLECULE TYPE: cDNA

10

(vi) ORIGINAL SOURCE:
 (A) ORGANISM: *Heliothis virescens*

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(vii) IMMEDIATE SOURCE:
 (B) CLONE: pSK19R

20

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

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(2) INFORMATION FOR SEQ ID NO: 3:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 2464 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: double
 - (D) TOPOLOGY: circular

- (ii) MOLECULE TYPE: cDNA

- (vi) ORIGINAL SOURCE:
 - (A) ORGANISM: Heliothis virescens

- (vii) IMMEDIATE SOURCE:
 - (B) CLONE: pSK16.1

40	(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:	
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(2) INFORMATION FOR SEQ ID NO: 4:

- 44 -

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:			
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	CCGCCCCGCC CGCCCGGCCG CCCGCCACCG TCTAGCGCGC CTCAGGAGAG AACGCTCATA	1980
	GACTGGCTAG TTTTAGTGAA GTGCACGGAC ACTGACGTCG ACGTGATCAA CCTATTTATA	2040
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40	GTGATGAGTC GTCCGCTGTC CACGTCGCCG TCACATGTT GTTCTGATG CACACGTGAG	2520
	GNCGGTTATC GTGTTTCATG GTTCCATCGT CCTGTGCCCG CGACCCCTCGA CTAAATGAGT	2580
	AATTTAATT ATTGCTGTGA TTACATTTA ATGTTGAT TATCTACCAT AGGGTGTAT	2640
45	AAGTGTGTCT TATTACAATA CAAAGTGTGT GTCGTCGATA GCTTCCACAC GAGCAAGCCT	2700
	TTTGTGTTAAG TGATTTACTG ACATGGACAC TCGACCCGGA ACTTC	2745

(2) INFORMATION FOR SEQ ID NO: 5:

50 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 575 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

55 (ii) MOLECULE TYPE: protein

60

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

- 46 -

	Met Ser Leu Gly Ala Arg Gly Tyr Arg Arg Cys Asp Thr Leu Ala Asp	
	1 5 10 15	
5	Met Arg Arg Arg Trp Tyr Asn Asn Gly Gly Phe Gln Thr Leu Arg Met	
	20 25 30	
	Leu Glu Glu Ser Ser Ser Glu Val Thr Ser Ser Ser Ala Leu Gly Leu	
	35 40 45	
10	Pro Pro Ala Met Val Met Ser Pro Glu Ser Leu Ala Ser Pro Glu Ile	
	50 55 60	
	Gly Gly Leu Glu Leu Trp Gly Tyr Asp Asp Gly Ile Thr Tyr Ser Met	
15	65 70 75 80	
	Ala Gln Ser Leu Gly Thr Cys Thr Met Glu Gln Gln Gln Pro Gln Pro	
	85 90 95	
20	Gln Gln Gln Pro Gln Gln Thr Gln Pro Leu Pro Ser Met Pro Leu Pro	
	100 105 110	
	Met Pro Pro Thr Thr Pro Lys Ser Glu Asn Glu Ser Met Ser Ser Gly	
	115 120 125	
25	Arg Glu Glu Leu Ser Pro Ala Ser Ser Val Asn Gly Cys Ser Thr Asp	
	130 135 140	
	Gly Glu Ala Arg Arg Gln Lys Lys Gly Pro Ala Pro Arg Gln Gln Glu	
30	145 150 155 160	
	Glu Leu Cys Leu Val Cys Gly Asp Arg Ala Ser Gly Tyr His Tyr Asn	
	165 170 175	
35	Ala Leu Thr Cys Glu Gly Cys Lys Gly Phe Phe Arg Arg Ser Val Thr	
	180 185 190	
	Lys Asn Ala Val Tyr Ile Cys Lys Phe Gly His Ala Cys Glu Met Asp	
	195 200 205	
40	Ile Tyr Met Arg Arg Lys Cys Gln Glu Cys Arg Leu Lys Lys Cys Leu	
	210 215 220	
	Ala Val Gly Met Arg Pro Glu Cys Val Val Pro Glu Asn Gln Cys Ala	
45	225 230 235 240	
	Met Lys Arg Lys Glu Lys Lys Ala Gln Arg Glu Lys Asp Lys Leu Pro	
	245 250 255	
50	Val Ser Thr Thr Val Asp Asp His Met Pro Pro Ile Met Gln Cys	
	260 265 270	
	Asp Pro Pro Pro Glu Ala Ala Arg Ile Leu Glu Cys Val Gln His	
	275 280 285	
55	Glu Val Val Pro Arg Phe Leu Asn Glu Lys Leu Met Glu Gln Asn Arg	
	290 295 300	
	Leu Lys Asn Val Pro Pro Leu Thr Ala Asn Gln Lys Ser Leu Ile Ala	
60	305 310 315 320	
	Arg Leu Val Trp Tyr Gln Glu Gly Tyr Glu Gln Pro Ser Glu Glu Asp	
	325 330 335	

- 47 -

Leu Lys Arg Val Thr Gln Ser Asp Glu Asp Asp Glu Asp Ser Asp Met
 340 345 350

5 Pro Phe Arg Gln Ile Thr Glu Met Thr Ile Leu Thr Val Gln Leu Ile
 355 360 365

Val Glu Phe Ala Lys Gly Leu Pro Gly Phe Ala Lys Ile Ser Gln Ser
 370 375 380

10 Asp Gln Ile Thr Leu Leu Lys Ala Cys Ser Ser Glu Val Met Met Leu
 385 390 395 400

Arg Val Ala Arg Arg Tyr Asp Ala Ala Thr Asp Ser Val Leu Phe Ala
 405 410 415

15 Asn Asn Gln Ala Tyr Thr Arg Asp Asn Tyr Arg Lys Ala Gly Met Ala
 420 425 430

20 Tyr Val Ile Glu Asp Leu Leu His Phe Cys Arg Cys Met Tyr Ser Met
 435 440 445

Met Met Asp Asn Val His Tyr Ala Leu Leu Thr Ala Ile Val Ile Phe
 450 455 460

25 Ser Asp Arg Pro Gly Leu Glu Gln Pro Leu Leu Val Glu Asp Ile Gln
 465 470 475 480

Arg Tyr Tyr Leu Asn Thr Leu Arg Val Tyr Ile Leu Asn Gln Asn Ser
 485 490 495

30 Ala Ser Pro Arg Gly Ala Val Ile Phe Gly Glu Ile Leu Gly Ile Leu
 500 505 510

Thr Glu Ile Arg Thr Leu Gly Met Gln Asn Ser Asn Met Cys Ile Ser
 515 520 525

Leu Lys Leu Lys Lys Arg Lys Leu Pro Pro Phe Leu Glu Glu Ile Trp
 530 535 540

40 Asp Val Ala Asp Val Ala Thr Thr Ala Thr Pro Val Ala Ala Glu Ala
 545 550 555 560

Pro Ala Pro Leu Ala Pro Ala Pro Pro Ala Arg Pro Ala Thr Val
 565 570 575

45 (2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
 50 (A) LENGTH: 948 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: double
 (D) TOPOLOGY: linear

55 (ii) MOLECULE TYPE: cDNA
 (vi) ORIGINAL SOURCE:
 (A) ORGANISM: Spodoptera exigua

60 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:
 AGGCCGGAGT GCGTGGTGCC AGAAAACCAG TGTGCAATGA AAAGGAAAGA GAAAAAGGCA

	CAAAGGGAAA AAGACAAGTT GCCAGTCAGT ACAACGACAG TGGATGATCA CATGCCTCCC	120
5	ATTATGCAGT GTGATCCACC GCCTCCAGAG GCGCAGAA TTCACGAGGT GGTGCCACGA	180
	TTCCTGAATG AAAAGCTAAT GGACAGGACA AGGCTAAGA ATGTGCCCCC TCACTGCCAA	240
	CCAGAAGTCC TTAATAGCGA GGCTGGTCTG GTACCAAGAA GGCTATGAAC AGCCATCAGA	300
10	AGAGGATCTA AAAAGAGTCA CACAGTCGGA TGAAGACGAA GAAGAGTCGG ACATGCCGTT	360
	CCGTCAGATC ACCGAGATGA CGATCCTCAC AGTGCAGCTC ATTGTTGAAT TCGCTAAGGG	420
15	CCTACCAGCG TTCGCAAAGA TCTCACAGTC GGATCAGATC ACATTATTAA AGGCCTGTT	480
	GAGTGAGGTG ATGATGTTGC GAGTAGCTCG GCGGTACGAC GCGGCGACAG ACAGCGTGT	540
	GTTCGCCAAC AACCAAGCGT ACACCCGCGA CAACTACCGC AAGGCAGGCA TGGCCTACGT	600
20	CATCGAGGAC CTGCTGCACT TCTGCCGGTG CATGTACTCC ATGATGATGG ATAACGTCCA	660
	CTATGCACTG CTCACTGCCA TCGTCATTTT CTCAGACCGA CCCGGGCTTG AGCTAACCT	720
25	GTTGGTGGAG GAGATCCAGA GATATTACCT GAACACGCTG CGGGTGTACA TCCTGAACCA	780
	GAACAGTCGG TCGCCGTGCT GCCCTGTCAT CTACGCTAAG ATCCTCGGCA TCCTGACGGA	840
	GCTGCGGACC CTGGGCATGC AGAACTCCAA CATGTGCATC TCACTCAAGC TGAAGAACAG	900
30	GAACGTGCCG CCGTTCTTCG AGGATATCTG GGACGTCTC GAGTAAAA	948

(2) INFORMATION FOR SEQ ID NO: 7:

35 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 319 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

40 (ii) MOLECULE TYPE: protein

45 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

	Arg Pro Glu Cys Val Val Pro Glu Asn Gln Cys Ala Met Lys Arg Lys	
	1 5 10 15	
50	Glu Lys Lys Ala Gln Arg Glu Lys Asp Lys Leu Pro Val Ser Thr Thr	
	20 25 30	
	Thr Val Asp Asp His Met Pro Pro Ile Met Gln Cys Asp Pro Pro Pro	
55	35 40 45	
	Pro Glu Ala Ala Arg Ile Leu Glu Cys Val Gln His Glu Val Val Pro	
	50 55 60	
60	Arg Phe Leu Asn Glu Lys Leu Met Glu Gln Asn Arg Leu Lys Asn Val	
	65 70 75 80	
	Pro Pro Leu Thr Ala Asn Gln Lys Ser Leu Ile Ala Arg Leu Val Trp	
	85 90 95	

Tyr Gln Glu Gly Tyr Glu Gln Pro Ser Glu Glu Asp Leu Lys Arg Val
100 105 110

5 Thr Gln Ser Asp Glu Asp Asp Glu Asp Ser Asp Met Pro Phe Arg Gln
115 120 125

Ile Thr Glu Met Thr Ile Leu Thr Val Gln Leu Ile Val Glu Phe Ala
130 135 140

10 Lys Gly Leu Pro Gly Phe Ala Lys Ile Ser Gln Ser Asp Gln Ile Thr
145 150 155 160

Leu Leu Lys Ala Cys Ser Ser Glu Val Met Met Leu Arg Val Ala Arg
15 165 170 175

Arg Tyr Asp Ala Ala Thr Asp Ser Val Leu Phe Ala Asn Asn Gln Ala
180 185 190

20 Tyr Thr Arg Asp Asn Tyr Arg Lys Ala Gly Met Ala Tyr Val Ile Glu
195 200 205

Asp Leu Leu His Phe Cys Arg Cys Met Tyr Ser Met Met Met Asp Asn
210 215 220

25 Val His Tyr Ala Leu Leu Thr Ala Ile Val Ile Phe Ser Asp Arg Pro
225 230 235 240

Gly Leu Glu Gln Pro Leu Leu Val Glu Glu Ile Gln Arg Tyr Tyr Leu
30 245 250 255

Asn Thr Leu Arg Val Tyr Ile Leu Asn Gln Asn Ser Ala Ser Pro Arg
260 265 270

35 Gly Ala Val Ile Phe Gly Glu Ile Leu Gly Ile Leu Thr Glu Ile Arg
275 280 285

Thr Leu Gly Met Gln Asn Ser Asn Met Cys Ile Ser Leu Lys Leu Lys
40 290 295 300

Lys Arg Lys Leu Pro Pro Phe Leu Glu Glu Ile Asp Trp Asp Val
305 310 315

CLAIMS

1. DNA comprising the sequence shown in Seq ID No. 2.
- 5 2. DNA comprising the sequence shown in Seq ID No. 3.
3. DNA comprising the sequence shown in Seq ID No. 4.
- 10 4. DNA comprising a sequence which shows 60% or more homology with the sequence shown in Seq ID No 1, 2 or 3.
5. DNA according to claim 4 wherein said homology is in the range of 65% to 99%.
- 15 6. DNA which hybridises to the sequence shown in Seq. ID No. 2, 3 or 4, and which codes for at least part of the *Heliothis* ecdysone receptor.
- 20 7. DNA which is degenerate as a result of the genetic code to the DNA of any one of claims 1 to 6 and which codes for a polypeptide which is at least part of the *Heliothis* ecdysone receptor.
8. DNA comprising part of the sequence shown in Seq ID No. 2, and which codes for at least part of the *Heliothis* ecdysone receptor ligand binding domain.
- 25 9. DNA comprising part of the sequence shown in Seq ID No. 3, and which codes for at least part of the *Heliothis* ecdysone receptor ligand binding domain.
10. DNA comprising part of the sequence shown in Seq ID No. 4, and which codes for at least part of the *Heliothis* ecdysone receptor ligand binding domain.
- 30 11. DNA comprising a sequence which shows 60% or more homology with the sequence of claim 8, 9 or 10.
12. DNA according to claim 11 wherein said homology is in the range of 65% to 99%.
- 35 13. DNA which hybridises to the DNA of any one of claims 8 to 12 and which codes for at least part of the *Heliothis* ecdysone receptor ligand binding domain.

14. DNA which is degenerate as a result of the genetic code to the DNA of any one of claims 8 to 12 and which codes for a polypeptide which is at least part of the *Heliothis* ecdysone receptor ligand binding domain.
- 5 15. DNA comprising part of the sequence shown in Seq ID No. 2, and which codes for at least part of the *Heliothis* ecdysone receptor DNA binding domain.
- 10 16. DNA comprising part of the sequence shown in Seq ID No. 3, and which codes for at least part of the *Heliothis* ecdysone receptor DNA binding domain.
17. DNA comprising part of the sequence shown in Seq ID No. 4, and which codes for at least part of the *Heliothis* ecdysone receptor DNA binding domain.
- 15 18. DNA comprising a sequence which shows 60% or more homology with the sequence of claim 15, 16 or 17.
19. DNA according to claim 18 wherein said homology is in the range of 65% to 99%.
- 20 20. DNA which hybridises to the DNA of any one of claims 15 to 19 and which codes for at least part of the *Heliothis* ecdysone receptor DNA binding domain.
- 25 21. DNA which is degenerate as a result of the genetic code to the DNA of any one of claims 15 to 19 and which codes for a polypeptide which is at least part of the *Heliothis* ecdysone receptor DNA binding domain.
22. DNA comprising part of the sequence shown in Seq ID No. 2, and which codes for at least part of the *Heliothis* ecdysone receptor transactivation domain.
- 30 23. DNA comprising part of the sequence shown in Seq ID No. 3, and which codes for at least part of the *Heliothis* ecdysone receptor transactivation domain.
24. DNA comprising part of the sequence shown in Seq ID No. 4, and which codes for at least part of the *Heliothis* ecdysone receptor transactivation domain.
- 35 25. DNA comprising a sequence which shows 60% or more homology with the sequence of claim 22, 23 or 24.

26. DNA according to claim 25 wherein said homology is in the range of 65% to 99%.
27. DNA which hybridises to the DNA of any one of claims 22 to 26 and which codes for at least part of the *Heliothis* ecdysone receptor transactivation domain.
5
28. DNA which is degenerate as a result of the genetic code to the DNA of any one of claims 22 to 26 and which codes for a polypeptide which is at least part of the *Heliothis* ecdysone receptor transactivation domain.
- 10 29. DNA comprising part of the sequence shown in Seq ID No. 2, and which codes for at least part of the *Heliothis* ecdysone receptor hinge domain.
30. DNA comprising part of the sequence shown in Seq ID No. 3, and which codes for at least part of the *Heliothis* ecdysone receptor hinge domain.
15
31. DNA comprising part of the sequence shown in Seq ID No. 4, and which codes for at least part of the *Heliothis* ecdysone receptor hinge domain.
32. DNA comprising a sequence which shows 60% or more homology with the sequence
20 of claim 29, 30 or 31.
33. DNA according to claim 32 wherein said homology is in the range of 65% to 99%.
34. DNA which hybridises to the DNA of any one of claims 29 to 33 and which codes for at least part of the *Heliothis* ecdysone receptor hinge domain.
25
35. DNA which is degenerate as a result of the genetic code of the DNA of any one of claims 29 to 33 and which codes for a polypeptide which is at least part of the *Heliothis* ecdysone receptor hinge domain.
30
36. DNA having part of the sequence shown in Seq ID No. 2, and which codes for at least part of the *Heliothis* ecdysone receptor carboxy terminal region.
37. DNA having part of the sequence shown in Seq ID No. 3, and which codes for at least part of the *Heliothis* ecdysone receptor carboxy terminal region.
35

38. DNA having part of the sequence shown in Seq ID No. 4, and which codes for at least part of the *Heliothis* ecdysone receptor carboxy terminal region.
- 5 39. DNA comprising a sequence which shows 60% or more homology with the sequence of claim 36, 37 or 38.
40. DNA according to claim 39 wherein said homology is in the range of 65% to 99%.
- 10 41. DNA which hybridises to the DNA of any one of claims 36 to 40 and which codes for at least part of the *Heliothis* ecdysone receptor carboxy terminal region.
42. DNA which is degenerate as a result of the genetic code of the DNA of any one of claims 36 to 40 and which codes for a polypeptide which is at least part of the *Heliothis* ecdysone receptor carboxy terminal region.
- 15 43. A polypeptide comprising the *Heliothis* ecdysone receptor or a fragment thereof, wherein said polypeptide is substantially free from other proteins with which it is ordinarily associated, and which is coded for by the DNA of any preceding claim.
- 20 44. A polypeptide comprising the amino acid sequence shown in Seq ID No. 4 or any allelic variant or derivative thereof.
45. A polypeptide comprising part of the amino acid sequence shown in Seq ID No. 4 or any allelic variant or derivative thereof, which sequence provides the *Heliothis* ecdysone receptor ligand binding domain.
- 25 46. A polypeptide comprising part of the amino acid sequence shown in Seq ID No. 4 or any allelic variant or derivative thereof, which sequence provides the *Heliothis* ecdysone receptor DNA binding domain.
- 30 47. A polypeptide comprising part of the amino acid sequence shown in Seq ID No. 4 or any allelic variant or derivative thereof, which sequence provides the *Heliothis* ecdysone receptor transactivation domain.
- 35 48. A polypeptide comprising part of the amino acid sequence shown in Seq ID No. 4 or any allelic variant or derivative thereof, which sequence provides the *Heliothis* ecdysone receptor hinge domain.

49. A polypeptide comprising part of the amino acid sequence shown in Seq ID No. 4 or any allelic variant or derivative thereof, which sequence provides the *Heliothis* ecdysone receptor carboxy terminal region.
5
50. A polypeptide according to any one of claims 44 to 49 wherein said derivative is a homologous variant which includes conservative amino acid changes.
51. DNA comprising the sequence shown in Seq ID No. 6.
10
52. DNA comprising a sequence which shows 60% or more homology with the sequence shown in Seq ID No. 6.
53. DNA according to claim 52 wherein said homology is in the range of 65% to 99%.
15
54. DNA which hybridises to the DNA sequence shown in Seq ID No. 6 and which codes for at least part of *Spodoptera* ecdysone receptor.
55. DNA which is degenerate as a result of the genetic code to the DNA of any one of
20 claims 51 to 54.
56. DNA comprising part of the sequence shown in Seq ID No. 6, and which codes for at least part of the *Spodoptera* ecdysone receptor ligand binding domain.
- 25 57. DNA comprising a sequence which shows 60% or more homology with the sequence of claim 56.
58. DNA according to claim 57 wherein said homology is in the range of 65% to 99%.
- 30 59. DNA which hybridises to the DNA of any one of claims 56 to 58 and which codes for at least part of the *Spodoptera* ecdysone receptor ligand binding domain.
60. DNA which is degenerate as a result of the genetic code to the DNA of any one of
35 claims 56 to 58 and which codes for at least part of the *Spodoptera* ecdysone receptor ligand binding domain.

61. DNA comprising part of the sequence shown in Seq ID No. 6, and which codes for at least part of the *Spodoptera* ecdysone receptor hinge domain.
- 5 62. DNA comprising a sequence which shows 60% or more homology with the sequence of claim 61.
63. DNA according to claim 62 wherein said homology is in the range of 65% to 99%.
- 10 64. DNA which hybridises to the DNA of any one of claims 61 to 63 and which codes for at least part of the *Spodoptera* ecdysone receptor hinge domain.
- 15 65. DNA which is degenerate as a result of the genetic code to the DNA of any one of claims 61 to 63 and which codes for at least part of the *Spodoptera* ecdysone receptor hinge domain.
66. A polypeptide coded for by the DNA of any one of claims 51 to 65.
- 20 67. A fusion polypeptide comprising the polypeptide of claim 45 or 50 (when dependent upon claim 45) and functionally linked to a DNA binding domain and a transactivation domain.
68. Recombinant DNA comprising the DNA of any one of claim 8 to 14 functionally linked to DNA encoding a DNA binding domain and a transactivation domain.
- 25 69. A fusion polypeptide according to claim 67 or recombinant DNA according to claim 68 wherein the DNA binding domain and/or transactivation domain is fungal, bacterial, plant or mammalian.
70. A fusion polypeptide or recombinant DNA according to claim 69 wherein the DNA binding domain is GAL4 or A1CR/A.
- 30 71. A fusion polypeptide or recombinant DNA according to claim 69 or 70 wherein the transactivation domain is VP16.
- 35 72. A fusion polypeptide or recombinant DNA according to claim 69 wherein the DNA binding domain and/or transactivation domain is from a steroid receptor superfamily member.

73. A fusion polypeptide or recombinant DNA according to claim 72 wherein the DNA binding domain and/or transactivation domain is from a glucocorticoid or a *Spodoptera* ecdysone receptor.

5

74. A recombinant DNA construct comprising recombinant DNA of any one of claims 68 to 73; and DNA which codes for a gene operably linked to a promoter sequence and a hormone response element, which is responsive to the DNA binding domain coded for by said recombinant DNA.

10

75. A fusion polypeptide comprising the polypeptide of claim 46 or 50 (when dependent upon claim 46) and functionally linked to a ligand binding domain and a transactivation domain.

15 76. Recombinant DNA comprising the DNA of any of claims 15 to 21 functionally linked to DNA encoding a ligand binding domain and a transactivation domain.

77. A fusion polypeptide according to claim 75 or recombinant DNA according to claim 76 wherein the ligand binding domain and/or transactivation domain is fungal, bacterial, plant or mammalian.

20

78. A fusion polypeptide or recombinant DNA according to claim 77 wherein the transactivation domain is VP16.

25 79. A fusion polypeptide or recombinant DNA according to claim 77 wherein the ligand binding domain and/or transactivation domain is from a steroid receptor superfamily member.

80. A fusion polypeptide or recombinant DNA according to claim 79 wherein the ligand binding domain and/or transactivation domain is from a glucocorticoid or *Spodoptera* ecdysone receptor.

30

81. A recombinant DNA construct comprising recombinant DNA of any one of claims 76 to 80; and DNA which codes for a gene operably linked to a promoter sequence and a hormone response element, which is responsive to the DNA binding domain coded for by said recombinant DNA.

35

82. A fusion polypeptide comprising the polypeptide of claim 47 or 50 (when dependent upon claim 47) and functionally linked to a ligand binding domain and a DNA binding domain.
- 5 83. Recombinant DNA comprising the DNA of any one of claims 22 to 28 functionally linked to DNA encoding a ligand binding domain and a DNA binding domain.
- 10 84. A fusion polypeptide according to claim 82 or recombinant DNA according to claim 83 wherein the ligand binding domain and/or DNA binding domain is fungal, bacterial, plant or mammalian.
85. A fusion polypeptide or recombinant DNA according to claim 84 wherein the DNA binding domain is GAL4 or A1CRA.
- 15 86. A fusion polypeptide or recombinant DNA according to claim 84 wherein the ligand binding domain and/or DNA binding domain is from a steroid receptor superfamily member.
- 20 87. A fusion polypeptide or recombinant DNA according to claim 86 wherein the ligand binding domain and/or DNA binding domain is from a glucocorticoid or *Spodoptera* ecdysone receptor.
- 25 88. A recombinant DNA construct comprising recombinant DNA of any one of claims 82 to 87; and DNA which codes for a gene operably linked to a promoter sequence and a hormone response element, which is responsive to the DNA binding domain coded for by said recombinant DNA.
- 30 89. A recombinant DNA construct comprising DNA according to any one of claims 1 to 7; and DNA comprising a sequence which codes for a gene operably linked to a promoter sequence and at least one hormone response element which is responsive to the DNA binding domain coded for by said DNA of any one of claim 1 to 7.
- 35 90. A recombinant DNA construct according to any one of claims 74, 81, 88 and 89 wherein said promoter sequence codes for a constitutive, spatially or temporally regulating promoter.

91. A recombinant DNA construct according to any one of claims 74, 81, 88 and 89 wherein there is more than one copy of the hormone response element.
92. A cell transformed with the DNA of any one of claims 1 to 42, and 51 to 65; the polypeptide of any one of claims 43 to 50; the fusion polypeptide of any one of claims 67, 70 to 73, 75, 77 to 80, 82 and 84 to 87; the recombinant nucleic acid of any one of claims 68 to 73, 76 to 80 and 85 to 87; or the recombinant DNA construct of any one of claims 74, 81, 88 and 89.
5
- 10 93. A cell according to claim 92 wherein said cell is a plant, fungal or mammalian cell.
94. A plant, fungus or mammal comprising the recombinant DNA construct of any one of claims 74, 81, 88 and 89.
- 15 95. A method of selecting compounds capable of being bound to an insect steroid receptor superfamily member comprising screening compounds for binding to said polypeptide of any one of claims 43 to 50 or the fusion polypeptide of any one of claims 67, 70 to 73, 75, 77 to 80, 82 and 84 to 87, and selecting said compounds exhibiting said binding.
20
96. A compound selected using the method of claim 95.
97. An agricultural or pharmaceutical composition comprising the compound of claim 96.
- 25 98. Use of the compound of claim 96 as an agrochemical or a pharmaceutical.
99. A method of producing a protein, peptide or polypeptide comprising introducing into the cell of claim 92, a compound which binds to the ligand binding domain in said cell.

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Sequence ID 1

Fig. 1.

1 TGGC AGG GGT GCA AGG AGT TCT TCA GGC GGA GTG TAA CCA AAA ATG
ACGC TCC CCA CGT TCC TCA AGA AGT CCT CCG ATT GGT TTT TAC

46 CAG TGT ACA TAT GCA AAT TCG GCC ATG CTT GCG AAA TGG ATA TGT
GTC ACA TGT ATA CGT TTA AGC CGG TAC GAA CGC TTT ACC TAT ACA

91 ATA TGC GGA GAA AAT GCC AAG AGT A
TAT ACG CCT CTT TTA CGG TTC TCA T

Fig.2.
Sequence ID: 2

3 1	9 ACT GGT GTT TTC ACC ACC ACA GAA AAG TGG TGT CTT TCC CGG AGA CGA GTA AAT TGA CCA CAA AAG CGA TTC TAG AGG ACG ACG GGT CGC GAC	15 46	21 GGT GGT GCT AAG AAG GTC ATC ATC TCC TGC TGC CCA GCG CTG CCA CCA CGA CGA TTC TAG TAG AGG ACG ACG GGT CGC GAC	27 91	TCC CAA CCT TGA AGC AGT ATG ACC CCT GTA CAA GCA ACC ACA GTT GGA ACT TCA TAC TGG GGA	33 TGG CGT CGT CGT TGG TGT CAA CCT TGA AGC AGT ATG ACC CCT GCA ACC ACA GTT GGA CGT GTT GGT TGA CGG AGC	39 136	TGT TCC AGT AGA GGT TGC GGA CCT GCA CAA CCA ACT GCC TCG ACA AGG TCA TCT CCA ACG CCT GCA CAA CCA ACT GCC TCG GAA TGT TCC AGT AGA GGT TGC GGA CCT GCA CAA CCA ACT GCC TCG	45 2/56	181	CTC TCG CTA AGG TCA TCC ATG ACA ACT TCG AGA TCA TTG AAG GAG AGC GAT TCC AGT AGG TAC TGT TGA AGC TCT AGT AAC TTC	226	GTC TGA CCA CTG TAC ACG CCA CCT CTG CCA CCC AGA AGA CAG CAG ACT ACT GGT GAC ATG TGC GGT GAC GGT GGG TCT TCT GTC	271	TGG ATG GAC CCT CTG GTA AAC TGT GGC GTG ATG GCC GTG GTG CTC ACC TAC CTG GGA GAC CAT TTG ACA CCG CAC TAC CGG CAC CAC GAG	316	AGC AGA ATA TCA TTC CCG CGG AAT TCC CCA GCC GCA GCT AGC TAA TCG TCT TAT AGT AAG GGC GCC TTA AGG GGT CGG CGT CGA TCG ATT
-------------	--	---------------	---	---------------	--	---	----------------	---	------------	-----	--	-----	--	-----	--	-----	--

Fig.2 i.

361 CCT GCA GCA GAC ACA ACC CCT ACC TTC CAT GCC GTT ACC AAT GCC
GGA CGT CGT CGT TGG TGG GGA TGG AAG GTA CGG CAA TGG TTA CGG

406 ACC GAC AAC ACC CAA ATC AGA AAA CGA GTC AAT GTC ATC AGG TCG
TGG CTG TTG TGG GRT TAG TCT TTT GCT CAG TTA CAG TAG TCC AGC

451 TGA GGA ACT GTC TCC AGC TTC GAG TGT AAA CGG CTG CAG CAC AGA
ACT CCT TGA CAG AGG TCG AAG CTC ACA TTT GCC GAC GTC GTG TCT

496 TGG CGA GGC GAG GCG GCA GAA GAA AGG CCC AGC GCC GAG GCA GCA
ACC GCT CCG CTC CGG CGT CTT CTC AGA GAC GGC GCT GTC TCG CGG CGT CGT

541 AGA AGA GCT ATG TCT TGT CTG CGG CGA CAG AGC CTC CGG ATA TCA
TCT TCT CGA TAC AGA ACA GCA GAC GGC GCT GTC TCG GAG GCC TAT AGT

586 CTA CAA CGC GCT CAC ATG TGA AGG GTG TAA AGG TTT CTT CAG GCG
GAT GTT GCG CGA GTG TAC ACT TCC CAC ATT TCC AAA GAA GTC CGC

631 GAG TGT AAC CAA AAA TGC AGT GTA CAT ATG CAA ATT CGG CCA TGC
CTC ACA TTG GTT TTT ACG TCA CAT GTA TAC GTT TAA GCC GGT ACG

676 TTG CGA AAT GGA TAT CTA TAT GCG GAG AAA ATG TCA GGA GTG TCG
AAC GCT TTA CCT ATA GAT ATA CGC CCC GTA CTC CGG GCT CAC AGC

721 GTT GAA GAA ATG TCT TGC GGT GGG CAT GAG GCC CGA GTG CGT GGT
CAA CTT CTT TAC AGA ACG CCA CGC CTC CGG GCT CAC GCA CCA

766 GCC GGA GAA CCA GTG TGCA AAT GAA AGC GAA AGA GAA AAA GGC GCA
CGG CCT CTT GGT CAC AGG TTA CTT TGC CTT TCT CTT TTT CCG CGT

361
451

Fig.2 ii.

4
56

811	GAG	GGA	AAA	AGA	CAA	ATT	GCC	CGT	CAG	TAC	GAC	GAC	AGT	AGA	CGA
	CTC	CCT	TTT	TCT	GTT	TAA	CGG	GCA	GTC	ATG	CTG	TCA	TCT	GCT	
856	TCA	CAT	GCC	TCC	CAT	GCA	ATG	TGA	CCC	TCC	GCC	CCC	AGA	GGC	
	AGT	GTA	CGG	AGG	GTA	CGT	TAC	ACT	GGG	CGT	GCT	CCA	CGG	TCT	CCG
901	CGC	TAG	AAT	TCT	GGA	ATG	TGT	GCA	GCA	GGT	GCC	AGC	ATT		
	GGG	ATC	TTA	AGA	CCT	TAC	ACA	CGT	GCT	GCT	CCA	CGG	TGC	TAA	
946	CCT	GAA	TGA	GAA	GCT	AAT	GGA	ACA	GAA	CAG	ATT	GAA	GAA	CGT	GCC
	GGG	CTT	ACT	CTT	CGA	TTA	CCT	TGT	CTT	GTC	TAA	CTT	CTT	GCA	CGG
991	CCC	CCT	CAC	TGC	CAA	TCA	GAA	GTC	GTT	GAT	CGC	AAG	GCT	CGT	GTG
	GGG	GGG	GTG	ACG	GTT	AGT	CTT	CAG	CAA	CTA	GCG	TTC	CGA	GCA	CAC
1036	GTA	CCA	GGA	AGG	CTA	TGA	ACA	ACC	TTC	CGA	GGA	AGA	CCT	GAA	GAG
	CAT	GGT	CCT	TCC	GAT	ACT	TGT	TGG	AAG	GCT	CCT	TCT	GGA	CTT	CTC
1081	GGT	TAC	ACA	GTC	GGA	CGA	GGA	CGA	AGA	CTC	GGA	TAT	GCC	GTT	
	CCA	ATG	TGT	CAG	CCT	GCT	CCT	GCT	GCT	TCT	GAG	CCT	ATA	CGG	CAA
1126	CCG	TCA	GAT	TAC	CGA	GAT	GAC	GAT	TCT	CAC	AGT	GCA	GCT	CAT	CGT
	GGC	AGT	CTA	ATG	GCT	CTA	CTG	CTA	AGA	GTG	TCA	CGT	CGA	GTA	GCA
1171	AGA	ATT	CGC	TAA	GGG	CCT	CCC	GGG	CTT	CGC	CAA	GAT	CTC	GCA	GTC
	TCT	TAA	GGG	ATT	CCC	GGA	GGG	CCC	GAA	GCG	GTT	CTA	GAG	CGT	CAG
1216	GGA	CCA	GAT	CAC	GTT	ATT	AAA	GGC	GTG	CTC	AAG	TGA	GGT	GAT	GAT
	CCT	GGT	CTA	GTG	CAA	TAA	TTT	CCG	CAC	GAG	TTC	ACT	CCA	CTA	CTA
1261	GCT	CCG	AGT	GGC	TCG	GCG	GTA	TGA	CGC	GGC	CAC	CGA	CAG	CGT	ACT
	CGA	GGC	TCA	CCG	AGC	CGG	CAT	ACT	GCG	CCG	GTG	GCT	GTC	GCA	TGA

Fig.2 iii.

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1306	GTT	CGC	GAA	CAA	CCA	GGC	GTA	CAC	TCG	CGA	CAA	CTA	CCG	CAA	GGC	
	CAA	GGC	CTT	GTT	GGT	CCG	CAT	GTC	AGC	GCT	GTT	GAT	GGC	GTC	CGG	
1351	AGG	CAT	GGC	GTA	CCT	CAT	CGA	GGG	CCT	GCT	GCA	CTT	CTG	TCG	GTG	
	TCC	GTA	CCG	CAT	GCA	GTA	GCT	CCT	GGG	CGA	CGT	GAA	GAC	AGC	CAC	
1396	CAT	GTA	CTC	CAT	GAT	GGA	TAA	CGT	GCA	TTA	TGC	GCT	GCT	TAC		
	GTA	CAT	GAG	GTA	CTA	CTA	CCT	ATT	GCA	CGT	AAT	ACG	CGA	CGA	ATG	
1441	AGC	CAT	TGT	CAT	CTT	CTC	AGA	CCG	GCC	CGG	GCT	TGA	GCA	ACC	CCT	
	TCG	GTA	ACA	GTA	GAA	GAG	TCT	GGC	GGG	GGG	GCC	CGA	ACT	CGT	TGG	GGG
1486	GTT	GCT	GGG	GGA	CAT	CCA	GAG	ATA	TTA	CCT	GAA	CAC	GCT	ACG	GGT	
	CAA	CCA	CCT	CCT	GTA	GGT	CTC	TAT	ATT	GGG	CTT	GTG	CGA	TGC	CCA	
1531	GTA	CAT	CCT	GAA	CCA	GAA	CAG	CGC	GTC	GCC	CCG	CGG	CGC	CGT	CAT	
	CAT	GTA	GGG	CTT	GGT	CTT	GTC	GGG	CAG	CGG	GGG	GGC	GGG	GCA	GTA	
1576	CTT	CGG	CGA	GAT	CCT	GGG	CAT	ACT	GAC	GGA	GAT	CCG	CAC	GCT	GGG	
	GAA	GCC	GCT	CTA	GGG	CCC	GTA	TGA	CTG	CCT	CTA	GGC	GTG	CGA	CCC	
1621	CAT	GCA	GAA	CTC	CAA	CAT	GTG	CAT	CTC	CCT	CAA	GCT	GAA	CAG		
	GTA	CGT	CTT	GAG	GTT	GTA	CAC	GTA	GAG	GGG	GTT	CGA	CTT	CTT	GTC	
1666	GAA	GCT	GCC	GCC	GTT	CCT	CGA	GGA	GAT	CTG	GGG	CGT	GGG	GGG	CGT	
	CTT	CGA	CGG	CGG	CAA	GGG	GCT	GCT	CCT	CTA	GAC	CCT	GCA	CCT	GCA	
1711	GGC	GAC	GAC	GGC	GAC	GCC	GGT	GGC	GGC	GGC	GGC	GGC	GGC	TCT		
	CCG	CTG	CTG	CCG	CTG	CCG	CCA	CCA	CCG	CCG	CCG	CCG	CCG	CCG	AGA	

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Fig.2 iv.

1756	AGC	CCC	CGC	CCC	GCC	CGC	CGG	GCC	CGC	CAC	CGT	CTA	GCG	CGC	
	TCG	GGG	GCA	GAT	CGC	GGC	GGC								
1801	CTC	AGG	AGA	GAA	CGC	TCA	TAG	ACT	GGC	TAG	TTT	TAG	TGA	AGT	GCA
	GAG	TCC	TCT	CTT	GCG	AGT	ATC	TGA	CCG	ATC	AAA	ATC	ACT	TCA	CGT
1846	CGG	ACA	CTG	ACG	TCG	ACG	TGA	TCA	ACC	TAT	TAA	TAA	GGA	CTG	CGA
	GCC	TGT	GAC	TGC	AGC	TGC	ACT	AGT	TGG	ATA	AAT	ATT	CCT	GAC	GCT
1891	ATT	TTA	CCA	CTT	AAG	AGG	GCA	CAC	CCG	TAC	CCC	ATT	TCG	TAC	GG
	TAA	AAT	GGT	GAA	TTC	TCC	CGT	GTG	GGC	ATG	GGC	TAA	AGC	ATG	CC

Total number of bases is: 1934.

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Fig.3.

The sequence shown below is that of psK16.1

Sequence ID3

3	9	15	21	27	33	39	45
1 CGC TGG TAT AAC AAC GGA CCA TTC CAG ACG CTG CGA ATG CTC GAG							
46 GAG AGC TCG TCT GAG GTG ACG TCG TCT TCA GCA CTG GGC CTG CCG							
91 CGG GCT ATG GTG ATG TCC CCG GAA TCG CTC GCG TCG CCC GAG ATC							
136 GGC GGC CTG GAG CTG TGG GGC TAC GAC GAT GGC ATC ACT TAC AGC							
181 ATG GCA CAG TCG CTG GGC ACC TGC ACC ATG GAG CAG CAG CCC							
TAC CGT GTC AGC GAC CCG TGG ACG TGG TAC CTC GTC GTC GGG							

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Fig.3 i.

226 CAG CCG CAG CAG CAG CCG CAG CAA CCC CTA CCT TCC ATG
 GTC GGC GTC GTC GTC GGC GTC GTC GTC GTC GTC GTC GTC GTC GTC
 271 CCG TTA CCA ATG CCA CCG ACA CCC AAA TCA GAA AAC GAG TCA
 GGC AAT GGT TAC GGT GGC TGT TGT GGG TTT AGT CTT TTG CTC AGT
 316 ATG TCA TCA CGT GAG GAA CTG TCT CCA GCT TCG AGT GTA AAC
 TAC AGT AGT CCA GCA CTC CTC GAC AGA GGT CGA AGC TCA CAT TTG
 361 GGC TGC AGC ACA GAT GGC GAG GCG AGG CGG CAG AAG AAA GGC CCA
 CCG ACG TCG TGT CTA CGG CTC CGC TCC GCC GTC TTC TTT CCG GGT
 406 GCG CCG AGG CAG CAA GAA GAG CTA TGT CTT GTC GGC GAC AGA
 CGC GGC TCC GTC GTT CTC GAT ACA GAA CAG ACG CCG CTG TCT
 451 GCC TCC GGA TAT CAC TAC AAC GCG CTC ACA TGT GAA GGG TGT AAA
 CGG AGG CCT ATA GTG ATG TTG CGC GAG TGT ACA CTT CCC ACA TTT
 496 GGT TTC TCC AGG CGG AGT GTA ACC AAA AAT GCA GTG TAC ATA TGC

Fig.3 ii.

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CCA	AAG	AAG	TCC	GCC	TCA	CAT	TGG	TTT	TTA	CGT	CAC	ATG	TAT	ACG	
541	AAA	TTC	GGC	CAT	GCT	TGC	GAA	ATG	GAT	ATC	TAT	ATG	CGG	AGA	AAA
	TTT	AAG	CCG	GTA	CGA	ACG	CTT	TAC	CTA	TAG	ATA	TAC	GCC	TCT	TTT
586	TGT	CAG	GAG	TGT	CGG	TTG	AAG	AAA	TGT	CTT	GCG	GTG	GGC	ATG	AGG
	ACA	GTC	CTC	ACA	GCC	AAC	TTC	TTT	ACA	GAA	CGC	CAC	CCG	TAC	TCC
631	CCC	GAG	TGC	GTG	CCG	GAG	AAC	CAG	TGT	GCA	ATG	AAA	CGG	AAA	
	GGG	CTC	ACG	CAC	CAC	GGC	CTC	TG	GTC	ACA	CGT	TAC	TTT	GCC	TTT
676	GAG	AAA	AAG	GCG	CAG	AGG	GAA	AAA	GAC	AAA	TTG	CCC	GTC	AGT	AGG
	CTC	TTT	TTC	CGC	GTC	TCC	CTT	TTT	CTG	TTG	TTT	AAC	GGG	CAG	TCA
721	ACG	ACA	GTA	GAC	GAT	CAC	ATG	CCT	CCC	ATC	ATG	CAA	TGT	GAC	CCT
	TGC	TGT	CAT	CTG	CTA	GTG	TAC	GGA	GGG	TAG	TAC	GTG	ACA	CTG	GGA
766	CCG	CCC	CCA	GAG	GCC	GCT	AGA	ATT	CTG	GAA	TGT	GTG	CAG	CAC	GAG
	GGC	GGG	GGT	CTC	CGG	CGA	TCT	TAA	GAC	CTT	ACA	CAC	GTC	GTG	CTC
811	GTG	GTG	CCA	CGA	TTC	CTG	AAT	GAG	AAG	CTA	ATG	GAA	CAG	AAC	AGA
	CAC	CAC	GGT	GCT	AAG	GAC	TTA	CTC	TTC	GAT	TAC	CTT	GTC	TTG	TCT
856	TTG	AAG	AAC	GTG	CCC	CCC	CTC	ACT	GCC	AAT	CAG	AAG	TCG	TTG	ATC
	AAC	TTC	TTG	CAC	GGG	GGG	GAG	TGA	CGG	TTA	GTC	TTC	AGC	AAC	TAG
901	GCA	AGG	CTC	GTG	TGG	TAC	CAG	GAA	GGC	TAT	GAA	CAA	CCT	TCC	GAG
	CGT	TCC	GAG	CAC	ACC	ATG	GTC	CTT	CCG	ATA	CTT	GTT	GGG	AGG	CTC
946	GAA	GAC	CTG	AAG	AGG	GTT	ACA	CAG	TCG	GAC	GAG	GAC	GAA	GAC	
	CTT	CTG	GAC	TTC	TCC	CAA	TGT	GTC	AGC	CTG	CTC	CTG	CTT	CTG	

Fig.3 iii.

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991	TCG	GAT	ATG	CCG	TTC	CGT	ATT	ACC	GAG	ATG	ACG	ATT	CTC	ACA
	AGC	CTA	TAC	GCG	AAG	GCA	GTC	TAA	TGG	CTC	TAC	TGC	TAA	GAG
1036	GTG	CAG	CTC	ATC	GTA	GAA	TTC	GCT	AAG	GGC	CTC	CCG	TTC	GCC
	CAC	GTC	GAG	TAG	CAT	CTT	AAG	CGA	TTC	CCG	GAG	GGC	CCG	AAG
1081	AAG	ATC	TCG	CAG	GAC	CAG	ATC	ACG	TTA	TTA	AAG	GCG	TGC	TCA
	TTC	TAG	AGC	GTC	AGC	CTG	GTC	TAG	TGC	AAT	TTC	CGC	ACG	AGT
1126	AGT	GAG	GTG	ATG	ATG	CTC	CGA	GTG	GCT	CGG	CGG	TAT	GAC	GCG
	TCA	CTC	CAC	TAC	TAC	GAG	GCT	CAC	CGA	GCC	GCC	ATA	CTG	CGG
1171	ACC	GAC	AGC	GTA	CTG	TTC	GGG	AAC	AAC	CAG	GCG	TAC	ACT	CGC
	TGG	CTG	TGG	CAT	GAC	AAG	CGC	TTG	TTG	GTC	CCG	ATG	TGA	GCG
1216	AAC	TAC	CGC	AAG	GCA	GGC	ATG	GGG	TAC	GTC	ATC	GAG	GAC	CTG
	TTG	ATG	GCG	TTC	CGT	CCG	TAC	CGC	ATG	CAG	TAG	CTC	CTG	GAC
1261	CAC	TTC	TGT	CGG	TGC	ATG	TAC	TCC	ATG	ATG	ATG	GAT	AAC	GTG
	GTG	AAG	ACA	GCC	ACG	TAC	ATG	AGG	TAC	TAC	TAC	TAC	CTA	TTG
1306	TAT	GCG	CTG	CTT	ACA	GCC	ATT	GTC	ATC	TTC	TCA	GAC	CGG	CCC
	ATA	CGC	GAC	GAA	TGT	CGG	TAA	CAG	TAG	AAG	AGT	CTG	GCC	GGG
1351	CTT	GAG	CAA	CCC	CTG	TTG	GTG	GAG	GAC	ATC	CAG	AGA	TAT	TAC
	GAA	CTC	GTC	GTT	GGG	GAC	AAC	CAC	CTC	CTG	TAG	GTC	TCT	ATA
1396	AAC	ACG	CTA	CGG	GTG	TAC	ATC	CTG	AAC	CAG	AAC	AGC	GCG	TGG
	TTC	TGC	GAT	GCC	CAC	ATG	TAG	GAC	TTG	GTC	TTC	TCG	CGC	AGC
1441	CGC	GGC	GCC	GTC	ATC	TTC	GGG	GAG	ATC	CTG	GGC	ATA	CTG	ACG

Fig.3 iv.

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1486	ATC	CGC	ACG	CTG	GGC	ATG	CAG	AAC	TCC	AAC	ATG	TGC	ATC	TCC	TCC
	TAG	GGG	TGC	GAC	CCG	TAC	GTC	TG	AGG	TG	TG	TAC	ACG	TAG	GAG
1531	AAG	CTG	AAC	AGG	AAG	CTG	CCG	CCC	TTC	CTC	GAG	GAG	ATC	TGG	
	TTC	GAC	TTC	TTG	TCC	TTC	GAC	GGC	GGC	AAG	GAG	CTC	CTC	TAG	ACC
1576	GAC	GTG	GCG	GAC	GTG	GCG	ACG	GGC	ACG	CCG	GGC	GTG	GGC	GGC	GAG
	CTG	CAC	CGC	CTG	CAC	CGC	TGC	TGC	CGC	TGC	GGC	CAC	CGC	CGC	CTC
1621	GCG	CCG	CCT	CTA	GCC	CCC	GCC	CCC	CCC	GCC	CCG	CCG	CCC	CCC	CCC
	CGC	GGC	CGC	GGG	GAT	CGG	GGG								
1666	ACC	GTC	TAG	CGC	GCC	TCA	GGA	GAG	AAC	GCT	CAT	AGA	CTG	GCT	AGT
	TGG	CAG	ATC	GGG	CGG	AGT	CCT	CTC	TTC	TTG	CGA	GTA	TCT	GAC	TCA
1711	TTT	AGT	GAA	GTG	CAC	GGG	CAC	TGA	CGT	CGA	CGT	GAT	CAA	CCT	ATT
	AAA	TCA	CTT	CAC	GTG	CCT	GTG	ACT	GCA	GCT	GCA	GCA	CTA	GTT	GAA
1756	TAT	AAG	GAC	TGC	GAA	TTT	TAC	CAC	TTA	AGA	GGG	CAC	ACC	CGT	ACC
	ATA	TTC	CTG	ACG	CTT	AAA	ATG	GTG	AAT	TCT	CCC	GTG	TGG	GCA	TGG
1801	CGA	TTT	CGT	ACG	TAT	TCG	GTG	ACC	GAC	GAT	GCA	GAG	CGT	GTG	
	GCT	AAA	GCA	TGC	ATA	AGC	CAC	TGG	CTG	CTG	CTA	CGT	CTC	GCA	CAC
1846	TAA	TGT	GAA	TAT	ATG	TGT	TGT	TGA	ACG	ATT	TGG	AGA	ATA	TAT	ATT
	ATT	ACA	CTT	ATA	TAC	ACA	ACA	ACT	TGC	TAA	ACC	TCT	TAT	ATA	TAA
1891	GCT	GTT	GCT	GTT	GGG	GGC	GGC	ACG	CCG	TCG	CCG	GTC	GGC	GGC	GAT
	CCA	CAA	CGA	CAA	CCC	GGG	GGG	TGC	GGC	AGC	GGC	CAG	CCG	CCG	CTA

Fig.3 V.

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1936	CGC	GGC	GCC	CGC	GGC	TTC	AGT	TTT	ATT	TCG	TTT	ACG	ACT	GAG	TTG
1981	GTC	ACT	CGG	ATA	CGA	CTG	TAT	GAT	AAG	ACT	TCG	TTC	GAT	AAG	TAC
CAG	TGA	GCC	TAT	GCT	GAC	ATA	CTA	TTC	TGA	AGC	AAG	CTA	TTC	ATG	
2026	ACC	TAC	TAA	ATT	ACA	CAT	ACG	TAC	GTA	GCT	TAC	GAG	AGT	TAT	TAG
TGG	ATG	ATT	TAA	TGT	GTA	TGC	ATG	CAT	CGA	ATG	CTC	TCA	ATA	ATC	
2071	AGA	CAA	AGA	ATA	TAA	GAA	GAA	GAT	GTT	TCT	ATT	GGG	TGA	AAA	GTT
TCT	GTT	TCT	TAT	ATT	CTT	CTT	CTA	CAA	AGA	TAA	CCC	ACT	TTT	CAA	
2116	GAT	AGT	TAT	GTT	TAT	TTA	CCA	AAA	TTA	ACA	ATA	ATA	CGT	TGA	TTA
CTA	TCA	ATA	CAA	ATA	AAT	GGT	TTT	AAT	TGT	TAT	TAT	GCA	ACT	AAT	
2161	ACC	TTT	CGA	GTA	TAA	TAT	TGT	GAT	GAG	TGC	TCC	GCT	GTC	CAC	GTC
TGG	AAA	GCT	CAT	ATT	ATA	ACA	CTA	CTC	AGC	AGG	CGA	CAG	GTG	CAG	
2206	GCC	GTC	ACA	TGT	TTG	TTT	CTG	ATG	CAC	ACG	TGA	GGN	GCG	TTA	TGC
CGG	CAG	TGT	ACA	AAC	AAA	GAC	TAC	GTG	TGC	ACT	CCN	CCG	AAT	AGC	
2251	TGT	TTC	ATG	GTT	CCA	TCG	TCC	TGT	GCC	CGC	GAC	CCT	CGA	CTA	AAT
ACA	AAG	TAC	CAA	GGT	AGC	AGG	ACA	CGA	CGG	GGC	CTG	GGG	GCT	GAT	TTA
2296	GAG	TAA	TTT	AAT	TTA	TTG	CTG	TGA	TTA	CAT	TTT	AAT	GTG	TTG	ATT
CTC	ATT	AAA	TTA	AAT	AAC	GAC	ACT	AAT	GTA	AAA	TTA	CAC	AAC	TAA	
2341	ATC	TAC	CAT	AGG	GTG	ATA	TAA	GTG	TGT	CTT	ATT	ACA	ATA	CAA	AGT
TAG	ATG	GTA	TCC	CAC	TAT	ATT	CAC	ACA	GAA	TAA	TGT	TAT	GTT	TCA	
2386	GTG	TGT	CGT	CGA	TAG	CTT	CCA	CAC	GAG	CAA	GCC	TTT	TGT	TTA	AGT

Fig.3 vi.

CAC ACA GCA GCT ATC GAA GGT GTG CTC GTT CGG AAA ACA AAT TCA
 2431 GAT TTA CTG ACA TGG ACA CTC GAC CCG GAA CTT C
 CTA AAT GAC TGT ACC TGT GAG CTG GGC CTT GAA G

Total number of bases is: 2464.

Fig.4.

Sequence ID 4

10 20 30 40 50 60
 | | | | |
 ACTCCGCTCTCTCACCTGCTGGATTGCTGGATTGCTGGATTGCTGGCC

 70 80 90 100 110 120
 | | | | |
 GCTCGAACGAGACTTCCGAGTCCCTATGGATTGGACGAAAGTCGAGACAGTAGCGA

 130 140 150 160 170 180
 | | | | |
 TTGGGTTCCGTTGAAACGTTGGTAGACGAGTGGCATGTCCTAGTCGCGTTAGAT

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Fig.4 i.

190 200 210 220 230 240
 | | | | |
 AGTTAGGCGGAGGAAAGTCAGTGAAGCCCTTCGGAGGGATGTCCCTCGGCCGTC

 250 260 270 280 290 300
 | | | | |
 GTGGATAACCGGAGGGTGTGACACCGCTCGCCGACATGAGACGCCGCTGGTATAACAACGGAC

 R G Y R C D T L A D M R R R W Y N N G

 310 320 330 340 350 360
 | | | | |
 CATTCCAGACGCTGCGAATGCTCGAGGAGGCTCGTCAAGGTGACGTCGTCAGCAC

 P F Q T L R M L E E S S S E V T S S S A

 370 380 390 400 410 420
 | | | | |
 TGGGCCTGCCGGCTATGGTGATGTCCCCGGAAATCGCTCGGAGATCGGCCG

 L G L P P A M V M S P E S L A S P E I G

Fig.4 ii.

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430	440	450	460	470	480														
 GCCCTGGAGGCTGTGGGGCTTACGACGATGGCATCACTTACAGCATGGCACAGTCGCTGGCCA																			
G	L	E	L	W	G	Y	D	D	G	I	T	Y	S	M	A	Q	S	L	G
490	500	510	520	530	540														
 CCTGCACCATTGGAGCAGCGAGCCAGCCAGCCAGCAGCAGCACACACCCC																			
T	C	T	M	E	Q	Q	P	Q	P	Q	Q	P	Q	P	Q	T	Q	P	
550	560	570	580	590	600														
 TACCTTCCATGCCGTTACCAATGCCACCGACAACACCCAAATCAGAAAACGAGTCAAATGT																			
L	P	S	M	P	L	P	M	P	P	T	T	P	K	S	E	N	E	S	M
610	620	630	640	650	660														
 CATCAGGGTCTGTGAGGAACCTGTCTCCAGCTTCGAGTGTAACCGGCTGCGAGCACAGATGGGG																			
S	S	G	R	E	E	L	S	P	A	S	S	V	N	G	C	S	T	D	G
670	680	690	700	710	720														
 AGGGAGGGCGAGAAGAAAGGCCAGGGCCAGGGAGGAGGCTATGTCTTGTCT																			
E	A	R	R	Q	K	K	G	P	A	P	R	Q	Q	E	E	L	C	L	V

Fig.4 III.

730 |
 GCGGGACAGAGCCTCCGGATATCACTACAACGGCTCACATGTGAAGGGTGTAAAGGTT
 C G D R A S G Y H Y N A L T C E G C K G
 740 |
 750 |
 760 |
 770 |
 780 |

790 |
 TCTTCAGGGGGAGTGTAAACCAAAAATGCAGTGTACATATGCCAATTGGCCATGCTTGCG
 F F R R S V T K N A V Y I C K F G H A C
 800 |
 810 |
 820 |
 830 |
 840 |

850 |
 AAATGGATATCTATATGCCGAGAAAATGTCAGGACTGTGGTTGAAGAAATGGCTTGCG
 E M D I Y M R R K C Q E C R L K K C L A
 860 |
 870 |
 880 |
 890 |
 900 |

910 |
 TGGGCATGAGCCCCGAGTGTGGTGGCTGCCGGAGAACCGAGTGTGCAATGAAACGGAAAGAGA
 V G M R P E C V V P E N Q C A M K R K E
 920 |
 930 |
 940 |
 950 |
 960 |

970 |
 AAAAGGGCGAGGGAAAGACAAAATTGCCCGTCAAGTAGCGACAGTAGACGATCACA
 K K A Q R E K D K L P V S T T V D D H

66

Fig.4 iv.

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1030	1040	1050	1060	1070	1080														
 TGCCTCCCATGCAATGTGACCCCTCCGCCAGGGCCCTAGAAATTCTGGAATGTG																			
M	P	P	I	M	Q	C	D	P	P	P	E	A	A	R	I	L	E	C	
1090	1100	1110	1120	1130	1140														
 TGCAGCACGGTGGTGCCACGATTCCTGAATGAGAACAGAACAGATTGA																			
V	Q	H	E	V	V	P	R	F	L	N	E	K	L	M	E	Q	N	R	L
1150	1160	1170	1180	1190	1200														
 AGAACGTGCCCTCACTGCCAATCAGAACGTCGTTGATCCAAAGGCTCGTGGTAC																			
K	N	V	P	P	L	T	A	N	Q	K	S	L	I	A	R	L	V	W	Y
1210	1220	1230	1240	1250	1260														
 AGGAAGGGCTATGAAACAACCTTCCGAGGAAGAACCTGAAAGAGGGTTACACAGTCGGACGAGG																			
Q	E	G	Y	E	Q	P	S	E	E	D	L	K	R	V	T	Q	S	D	E

Fig.4 V.

1270 1280 1290 1300 1310 1320
 | | | | |
 ACGACGAAGACTCGGATATGCCGTTCCGTCAAGATTACCGAGATGACGATTCTCACAGTGC

 D D E D S D M P F R Q I T E M T I L T V

 1330 1340 1350 1360 1370 1380
 | | | | |
 AGCTCATCGTAGAATTGGCTAAAGGGCCCTCCGGCTTCGCCAAGATCTCGCAGTGC

 Q L I V E F A K G L P G F A K I S Q S D

 1390 1400 1410 1420 1430 1440
 | | | | |
 AGATCACGTTATTAAAGGGCGTGTCAAGTGAGGTGATGATGCTCCGAGTGGCTCGCCGGT

 Q I T L L K A C S S E V M M L R V A R R

 1450 1460 1470 1480 1490 1500
 | | | |
 ATGACGGGCCACCGACAGCGTACTGTTGCGAACAAACCAGGGTACACTCGCGACAACT

Fig.4 vi.

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1510	1520	1530	1540	1550	1560
ACCGCAAGGCAGGCATGGCGTACGTCAATCGAGGACCTGCACTTGCTCGGGCATGTC					
Y R K A G M A Y V I E D L L H F C R C M					
1570	1580	1590	1600	1610	1620
ACTCCATGATGGATAACGTGCATTATGCCGTGCTTACAGGCCATTGTCATCTTCTCAG					
Y S M M D N V H Y A L L T A I V I F S					
1630	1640	1650	1660	1670	1680
ACCGGCCGGCTTGAGCAACCCCTGTTGGAGGAGATCCAGAGATATTACCTGAACA					
D R P G L E Q P L L V E E I Q R Y Y L N					
1690	1700	1710	1720	1730	1740
CGCTACGGGTGTACATCCTGAACCAACAGCGCGTGCCTCGCCCCGGCGCGTCATCTTCG					
T L R V Y I L N Q N S A S P R G A V I F					

Fig.4 vii.

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1750	1760	1770	1780	1790	1800
 GGGAGATCCGGCATACTGACGGAGATCCGCACGGCTGGCATGGAGAACCTCCAAACATGTC					
G E I L G I L T E I R T L G M Q N S N M					
1810	1820	1830	1840	1850	1860
 GCATCTCCCTCAAGCTGAAGAACAGGAAGCTGCCGCCGTCCCTCGAGGAGATCTGGGACCG					
C I S L K L K N R K L P P F L E E I W D					
1870	1880	1890	1900	1910	1920
 TGGGGACGTGGCGACCGACGGGACGCCGGTGGCGGGAGGGCCCTAGGCC					
V A D V A T T A T P V A A E A P A P L A					
1930	1940	1950	1960	1970	1980
 CCGGCCCCGGCCGCCACCGTCTAGCGGCCCTCAGGAGAGAACCGCTCATA					
P A P P A R P P A T V -					
1990	2000	2010	2020	2030	2040
 GACTGGCTAGTTTAGTGAAGTGCACGGGACACTGACGTGACGTGATCAAACCTATTATA					

Fig.4 viii.

2050 | 2060 | 2070 | 2080 | 2090 | 2100
 AGGACTGCGAATTTACCACTTAAGAGGGCACACCCGTACCGATTCGTACGTATTCGG
 2110 | 2120 | 2130 | 2140 | 2150 | 2160
 TGACCGACGACGATGCCAGAGCGCTGTGTAATGTGAATATTGTTGAAACGATTGGA
 2170 | 2180 | 2190 | 2200 | 2210 | 2220
 GAATATATAATTGGTGTGTTGCTGGTGGGGCCGCACGCCGTGCCGGTTCGGGGATCGCG
 2230 | 2240 | 2250 | 2260 | 2270 | 2280
 GCGCCGGCTTCAGTTTACGACTTGAGTTGACTCGGATAACGACTGACTGT
 2290 | 2300 | 2310 | 2320 | 2330 | 2340
 ATGATAAGGACTTCGTTCGATAAGTACACCTACTAAATTACACATAACGTACGGTAGCTACG
 2350 | 2360 | 2370 | 2380 | 2390 | 2400
 AGAGTTATTAGAGACAAAGAAATAAGAAGAAGATGTTCTATTGGGTGAAAAGTTGATA

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Fig.4 ix.

2410 2420 2430 2440 2450 2460
 | | | | | |
 GTTATGTTTACCAAAATTAAACAATAAACCGTTGATTAAACCTTTCGAGTATAATTATT

 2470 2480 2490 2500 2510 2520
 | | | | | |
 GTGATGAGTCGTCCGGCTGTGCCACGTCGGTCACATGTTGTTCTGATGCCACACGGTAG

 2530 2540 2550 2560 2570 2580
 | | | | | |
 GNGCGTTATCCTGTTCATGGTTCCATCGTCCCTGGCACCCCTGACTAAATGAGT

 2590 2600 2610 2620 2630 2640
 | | | | | |
 AATTAAATTATTGCTGTGATTACATTAAATGTTGATTCTACCATAGGGTGATAT

 2650 2660 2670 2680 2690 2700
 | | | | | |
 AAGTGTGCTTATTACAATAAACAAAGTGTGTCGATAGCTTCCACACGGAGCAAAGCCT

 2710 2720 2730 2740
 | | | |
 TTTGTTAAGTGATTACTGACATGGACACTCGACCCGGAACTC

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Sequence I.D. 5

Fig. 5.

BmECR	MREVNDNNVS	10	
M _s ECR	-----		
H _v ECR	-----	1	
C _t ECR	-----		
A _a ECR	-----		
D _m ECR	-----		
BmECR	FALNGRADEWCMSVTRLDLVREREKSEVKAYVGGCPSSVITDAGAYDALFD	60	
M _s ECR	-----		
H _v ECR	-----		DTLAD
C _t ECR	-----		
A _a ECR	-----		
D _m ECR	-----		
BmECR	M-RRRWSNNGGFP-LRMLEESSEVTSSSA-LGLPPAMVMSPESLASPEY	107	
M _s ECR	M-RRRWSNNGCFP-LRMFEESSEVTSSSA-FGMPAAVMSPESLASPEY	47	
H _v ECR	M-RRRWYNNNGGFQTLRMLLEESSEVTSSSA-LGLPPAMVMSPESLASPEY	64	
C _t ECR	M-K-----TENLIVTT-VKVEPLNYASQSF	23	
A _a ECR	MMKRRWSNNGGFTALRMLDDSSSEVTSSSAAL--GMTMSPNSLGSPNY	46	
D _m ECR	M-KRRWSNNGGF--MRLPEESESSEVTSSSNGLVLPSGVNMSPSSLDSHADY	47	

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Fig.5 i.

BmECR	GALEIW--	SY-----	114
MsECR	GGLEIW--	SY-----	55
HVECR	GGLEIW--	GY-----	72
CtECR	GDNNI-----	YGGAT-----	33
AaECR	DELEIW--	SYEDNAYNGHSV--	78
DmECR	CDNDKWLGNESGSFGGSNGHGLSQQQQSVITLAMHGCSSSTLPAQTTIIP	24/86	97
BmECR		DDGITY-----	121
MsECR		DETMTN-----	61
HVECR		DDGIT-----	77
CtECR		KKQRLESDETMINH-----	46
AaECR		ANNLJLMNG IIVGNNNL-----	98
DmECR	INGNANGNGGSTNQYVPGATNLGALANGMLNGGFNIGMQQQIQNGHGLIN		147
BmECR	NTAQSLLLGACNMQQQQQLQP-----	QQPHPAPPTLPTMP-----	154
MsECR	YPAQSLLLGACNAQQQQQQ-----	QQQOPSAQPLPSMP-----	94
HVECR	YSMAQSLGTCTMEQQQQQP-----	QQOPQQTQPLPSMP-----	114
CtECR	NQTNMNLLESSNMNHNTIS-----	GFPSPDVNYEAYSPNSKL-----	86
AaECR	MASQAVQANANSIQHIVGN-----	DDGN-----	134
DmECR	STTPSTPTPLHLQONLGGAGGGGIGGGMGILHHANGTPNGLIGVVGCCCC		197
BmECR	LPMPPPTPKSENEMSSGREELS PASSINGCSADA--D	190	
MsECR	LPMPPPTPKSENEMSSGREELS PASSINGCSTDG--E	130	
HVECR	LPMPPPTPKSENEMSSGREELS PASSVNGCSTDG--E	146	
CtECR	MSVHMGDG-----	K	98
AaECR	IIQNTLMNTPRSESVNNSISSGREDLSPSSSLNGYT--DGSD	173	
DmECR	VGLGVGGGGVGGLGMQHTPRSDVNNSISSGRDDLSBPSSSLNGYSANESCD		247

BmECR	ARRQKKGPAPRQQEELCLVCGDRASGYHYNALTCLEGCKGFFRRSVTKNAV	240
MsECR	PRRQKKGPAPRQQEELCLVCGDRASGYHYNALTCLEGCKGFFRRSVTKNAV	180
HvECR	ARROKKGPAPRQQEELCLVCGDRASGYHYNALTCLEGCKGFFRRSVTKNAV	196
CtECR	KSSSKKGPVPRQQEELCLVCGDRASGYHYNALTCLEGCKGFFRRSVTKNAV	148
AaECR	AKKQKKGPPTPRQQEELCLVCGDRASGYHYNALTCLEGCKGFFRRSVTKNAV	223
DmECR	AKKSKKGPAPRVQEQEELCLVCGDRASGYHYNALTCLEGCKGFFRRSVTKNAV	297
	*****	*****
BmECR	YICKFGHACEMDMYMRRKCQECLIKKCLAVGMRPECVQIPEPS-KNKDRQR	289
MsECR	YICKFGHACEMDMYMRRKCQECLIKKCLAVGMRPECVVPPESTCKNKRKEK	230
HvECR	YICKFGHACEMDIYMRRKCQECLIKKCLAVGMRPECVVPENQCAIKRKEK	246
CtECR	YCCCKFGHECEMDMYYMRRKCQECLIKKCLAVGMRPECVVPENQCAIKRKEK	198
AaECR	YCCCKFGHACEMDMYMRRKCQECLIKKCLAVGMRPECVVPENQCAIKRKEK	273
DmECR	YCCCKFGGRACEMDMYMRRKCQECLIKKCLAVGMRPGCVVPGNQCAMRREK	347
	*****	*****
BmECR	QKKDKGILLPVSTTTV-----EDHMPPIMQC	315
MsECR	EAQREKDQLPVPSTTTV-----DDHMPPAIMQC	256
HvECR	KAQREKDQLPVPSTTTV-----DDHMPPIMQC	272
CtECR	KAQKEKDVKPGIVGSNTSSSLINQSLNNNGSLKMLEISYREELLQQLMKC	248
AaECR	KAQKEKDVKQTNA-----VSTTNSTY-RS-----EILPTLMKC	306
DmECR	KAQKEKDMMTSPSSQHGNGSLASGGGQDFVKK-----EILD-LMTC	389
	*****	*****
BmECR	DPPPPEAARI-----HEVVPRYLSEKLMEQNRQKNIPPLSANQKSLIARL	360
MsECR	DPPPPEAARI-----HEVVPRFLTEKIMEQNRLKVNVTPLSANQKSLIARL	301
HvECR	DPPPPEAARILECVQHEVVPRFLNEKLMEQNRLKVNVPPLTANQKSLIARL	322
CtECR	DPPPHPMQQL-----PEKLMENRAKGTPOLTANQAVVIYKL	286
AaECR	DPPPHQAIPLL-----PEKLLQENRLRNIPLLTANQMAVVIYKL	344
DmECR	EPPQHATIPLL-----PDEILAKCQARNIPLSNTYNQLAVITKL	427
	*****	*****

Fig.5 ii.

BmECR	VWYQEGYEQPSDEDLKRVTQTWQ-	SDEEDEESDLPFRQITEMTILTQLI	409
MSECR	VMYQEGYEQPSEEDLKRVTQTWQLEEEEEEETDMMPFRQITEMTILTQLI	351	
HVECR	VWYQEGYEQPSEEDLKRVTQS---	368	
CtECR	-DEDDEDSDMPFRQITEMTILTQLI	334	
AaECR	IWYQDGYEQPSEEDLKRITTE--LEEEDQEHEANFRYITEVTLTVQLI	392	
DmECR	IWYQDGYEQPSEEDLKRIMIG--SPNEEEDQHDVHFRHITEITILTQLI	474	
	IWYQDGYEQPSEEDLRRIM-S--QPDENESQTDVSFRHITEITILTQLI		

BmECR	VEFAKGLPFGFSKISQSDQITLLKACSSSEVMMLRVARRYDAASDSVLFANN VEFAKGLPFGFSKISQSDQITLLKACSSSEVMMLRVARRYDAATDSVLFANN VEFAKGLPFGFAKISQSDQITLLKACSSSEVMMLRVARRYDAATDSVLFANN VEFAKGLPAFIKIPQEDQITLLKACSSSEVMMLRMARRYDHDSDSILFANN VEFAKGLPAFTKIPQEDQITLLKACSSSEVMMLRMARRYDAATDSILFANN VEFAKGLPAFTKIPQEDQITLLKACSSSEVMMLRMARRYDHSSDSIFFANN
MsECR	*****
HvECR	*****
CtECR	*****
AaECR	*****
DmECR	*****

BmECR	KAYTRDNYRQGGMAYVIEDLLHFCRCMFAMGMDNTFHALLTAIVIFS	509
MSECR	QAYTRDNYRKAGMSYVIEDLLHFCRCMYSMSMDNTHYALLTAIVIFS	451
HVECR	QAYTRDNYRKAGMAYVIEDLLHFCRCMYSMMDNHYALLTAIVIFS	468
CtECR	TAYTKQTYQLAGMEETIDDLLHFCRCQMYALSIDNVETALLTAIVIFS	434
AaECR	RSYTRDSYRMAGMADTIEDLLHFCRCQMFSLTVDNVEYALLTAIVIFS	492
DmECR	RSYTRDSYKMGADMNIEDLLHFCRCQFMSMKVDNVEYALLTAIVIFS	574

BmECR	GLEQPSLVEEIQRYYLNTLRIYIINQNSASSRCAVIYGRILSVLTTELRTL	559
MsECR	GLEQPLLVEEIQRYYLKTLRVYIINQNSASPRCAVLFGKILGVLTTELRTL	501
HvECR	GLEQPLLVEDIQRYYLNTLRLRVYIINQNSASPRGAVIFGEILGILTEIRTL	518
CtECR	GLEKAEMVDIIQSYYTETLKVYIVRDHGGECSRCSVFAKLIGILTTELRTM	484
AaECR	GLEQAELVEHIQSYYIDTLRIYIILNRHAGDPKCSVIFAKLSSILTELRTL	542
DmECR	GLEKAQLVEAIQSYYIDTLRITIILNRHGDMSLVFYAKLSSILTELRTL	624

Fig.5 iii.

BmECR	GTQNSNMCISLKLKNRKLPPFLEEIWDAEVARR-----	593
MSECR	GTQNSNMCISLKLKNRKLPPFLEEIWDAEVSTT-----	535
HVECR	GMQNNSNMCISLKLKNRKLPPFLEEIWDAADVATT-----	552
CtECR	GNLNSEMCFSSLKLRNRKLPRFLEEVWDVGDVNNQTTATTNTENIVRERIN	534
AaECR	GNQNSEMCFSSLKLRNRKLPRFLEEIWVDVQDIPPSMQAQMHSHGTQSSS--	590
DmECR	GNQNAEMCFSSLKLRNRKLPKFLEEIWDVHAIPPSVQSHLQITQEEDERLE	674
	* * . * * * * . * * * * . * * * * . * * * .	
BmECR	-----	593
MSECR	-----	535
HVECR	-----	552
CtECR	-----	536
AaECR	-----	632
DmECR	RAERMRAVGGAITAGIDCDSASTSAAAAAQHQPQPQPPQPSLTQND	724
BmECR	-----	586
MSECR	-----	606
HVECR	-----	556
CtECR	-----	575
AaECR	-----	536
DmECR	-----	645
BmECR	HPTV-----	606
MSECR	QP---TPGVAAQVTPIVVVDNPAA-----	556
HVECR	-----	575
CtECR	-ATPVAAEAAPPLAPAPPAPPATV-----	536
AaECR	-----	V
DmECR	QQHQQQ----HSQLQQ-----V	774
	SQHQTQPQLQPQLPQLQQLQPQLQTQLQPQIQPQPQLLPVSAPV	
BmECR	-----	606
MSECR	-----	556
HVECR	-----	575
CtECR	-----	536
AaECR	-----	663
DmECR	HANGSGGGSNNSSSG-----	824
	PASVATAPGSLSAVSTSSEYMGGSAAIGPITPATTSSITAAVTASSTTS	

Fig. 5 iv.

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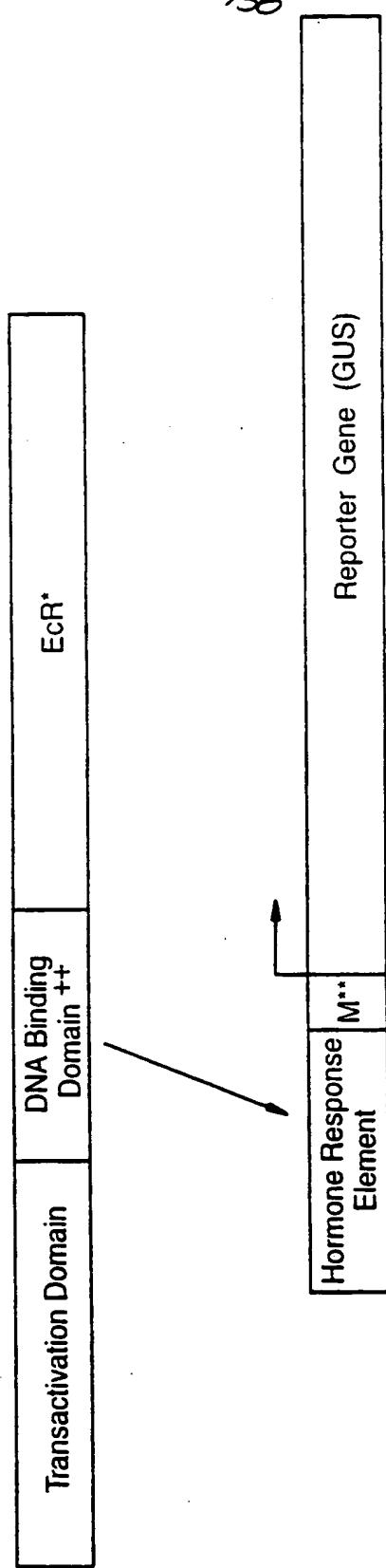
Fig.5 v.

BmECR	606
M _s ECR	556
H _v ECR	575
C _t ECR	536
A _a ECR	675
D _m ECR	874

BmECR	606
M _s ECR	556
H _v ECR	575
C _t ECR	536
A _a ECR	675
D _m ECR	878

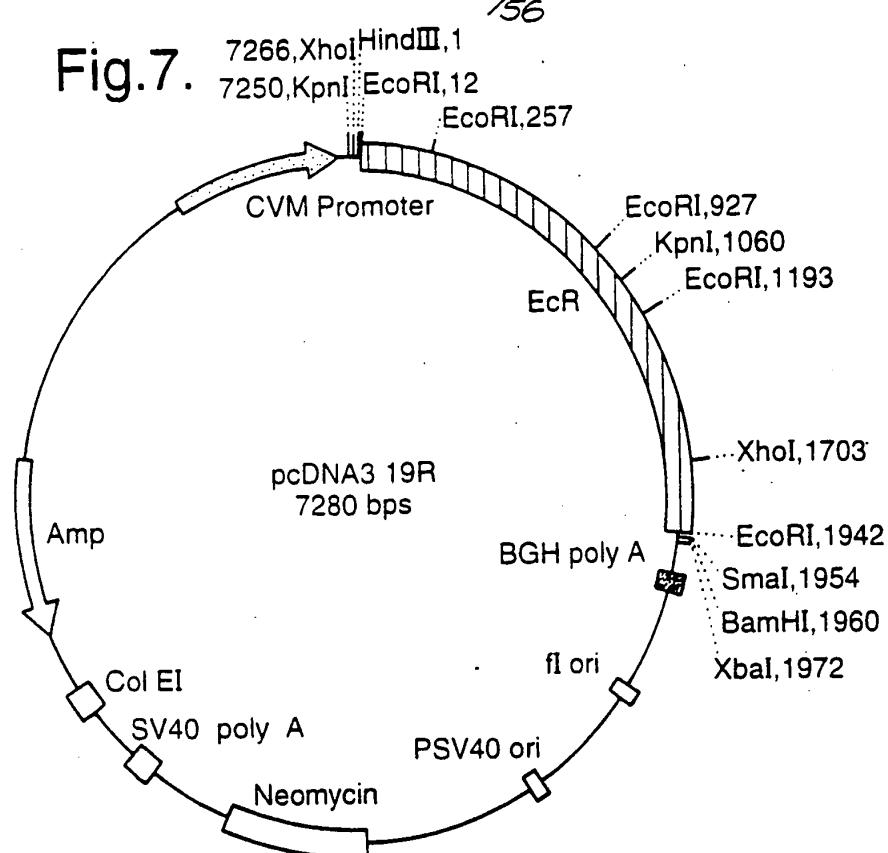
Fig.6.

Chemical



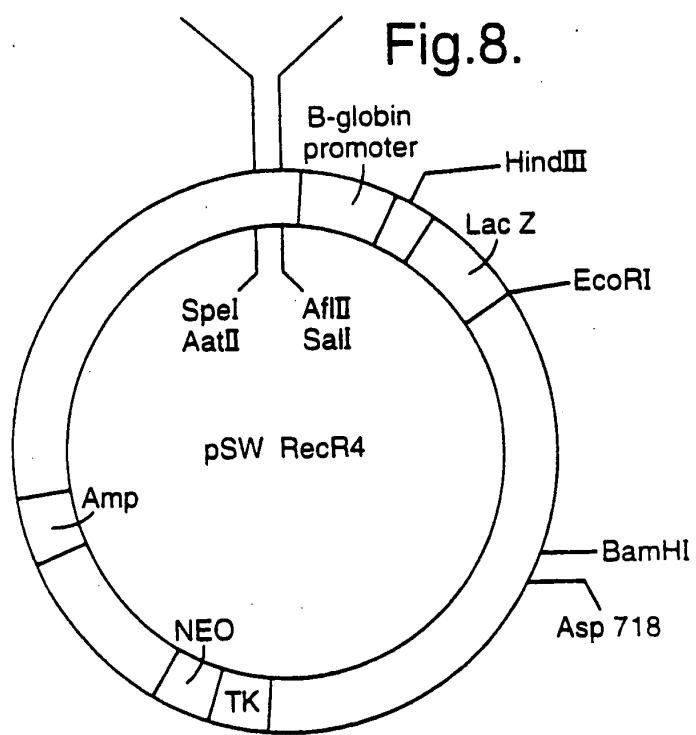
- ++ Glucocorticoid receptor DNA binding and transactivation domains
- * Insect ecdysone ligand binding domain
- ** Minimal 35S CaMV promoter

Fig.7.



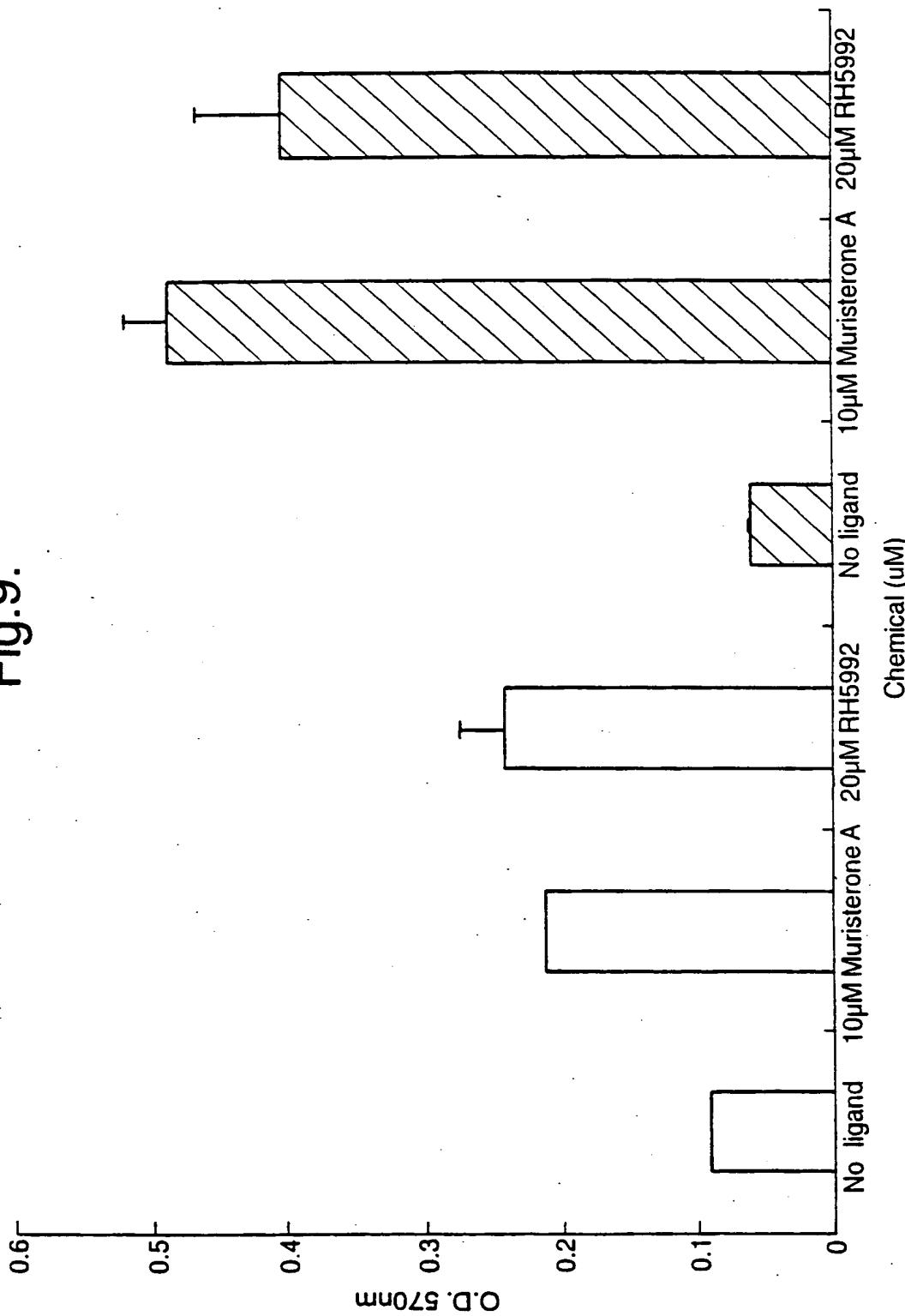
Response Element for HecR → → →

Fig.8.



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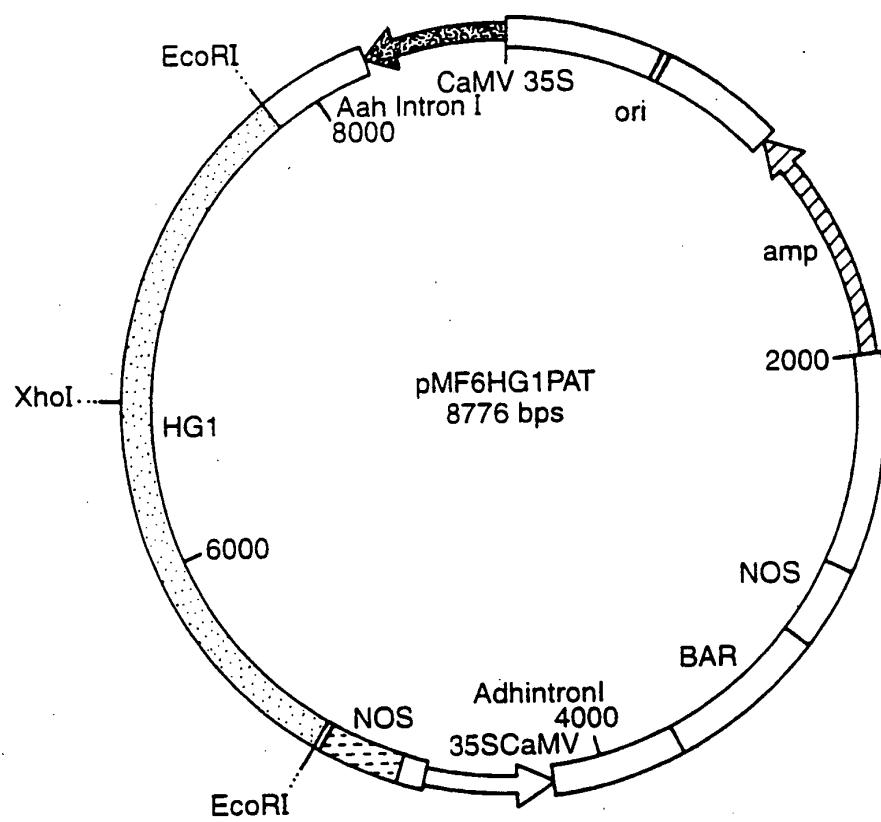
Fig. 9.



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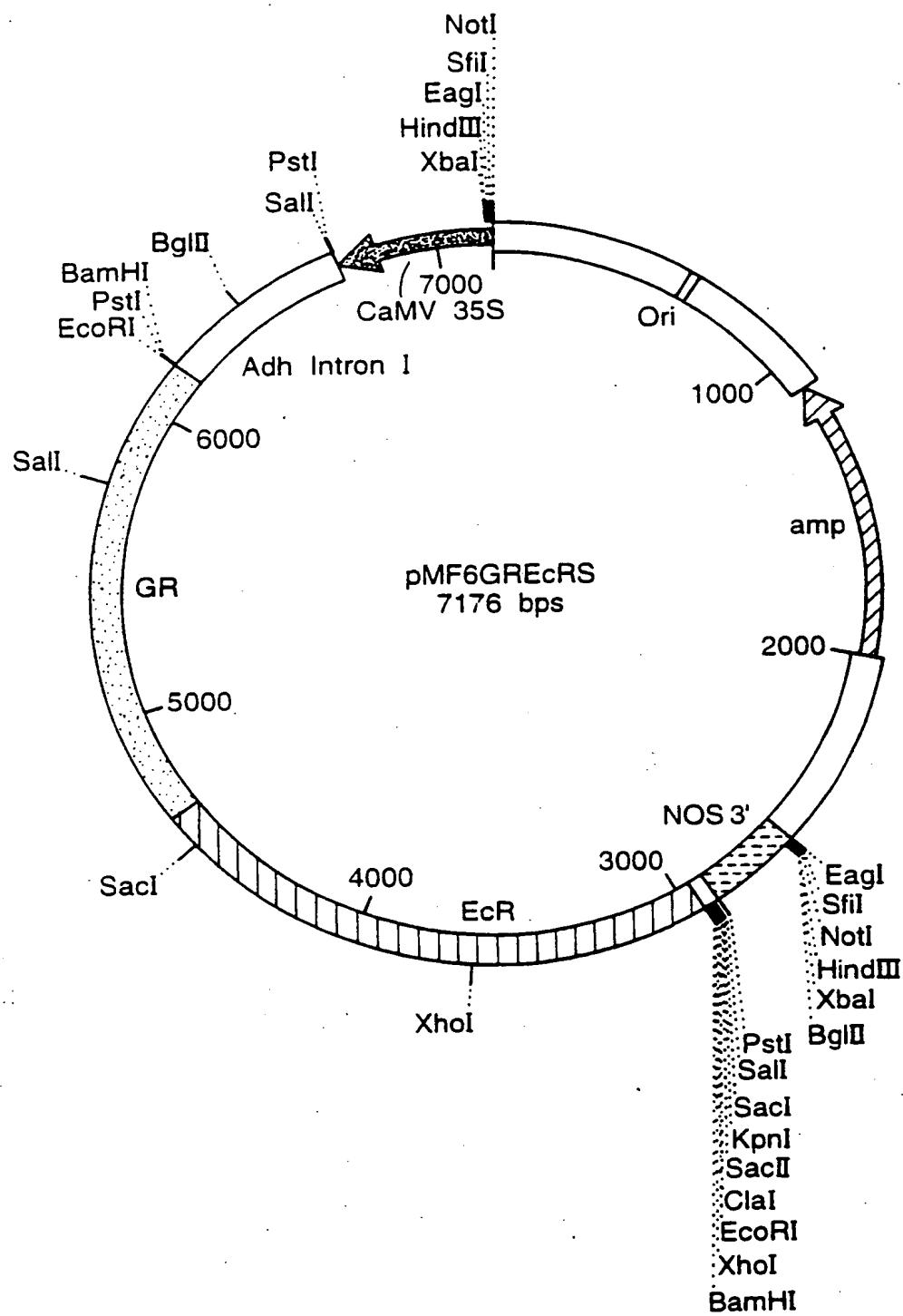
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Fig.10.



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Fig. 11.



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Fig. 12.

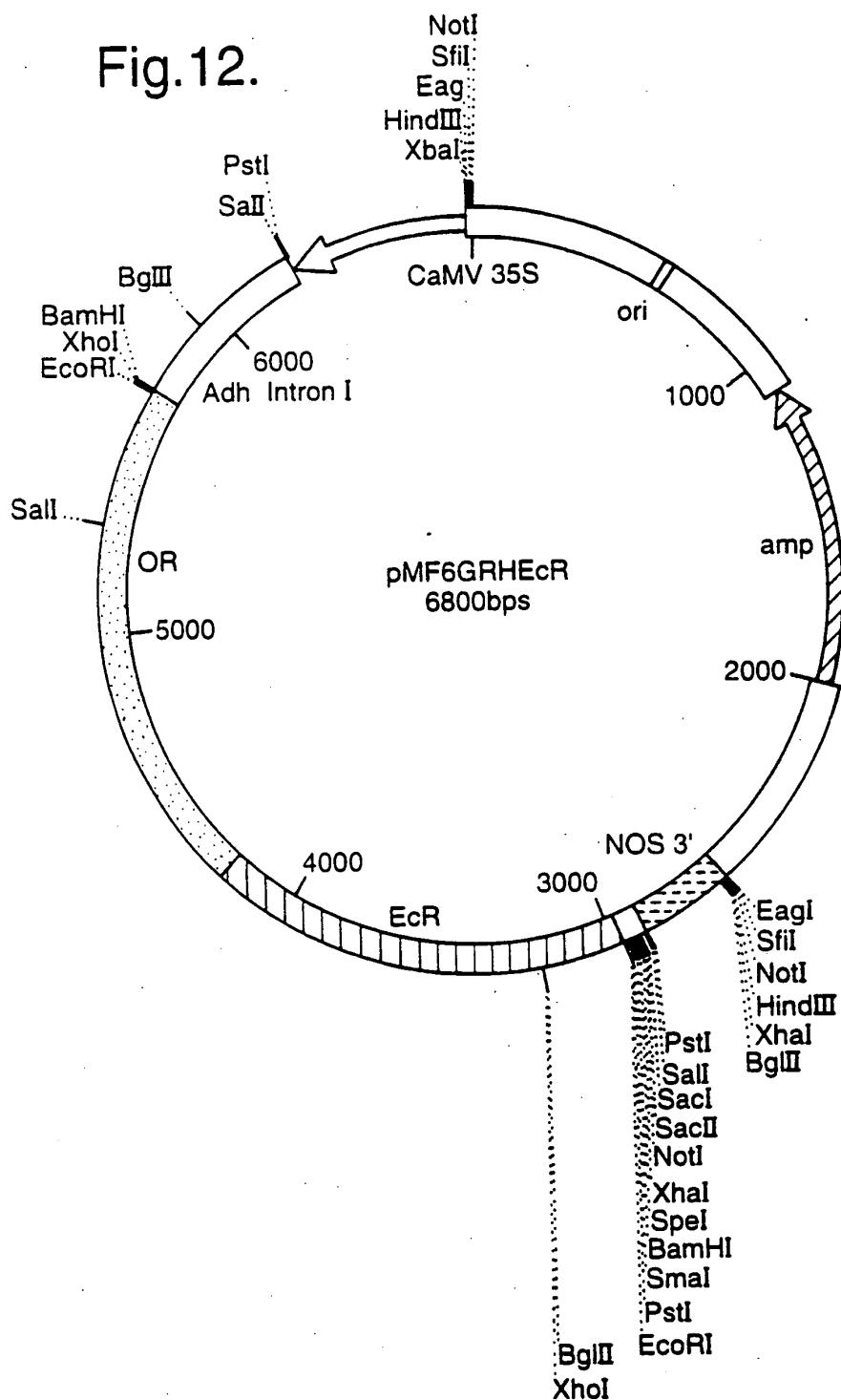


Fig.13.

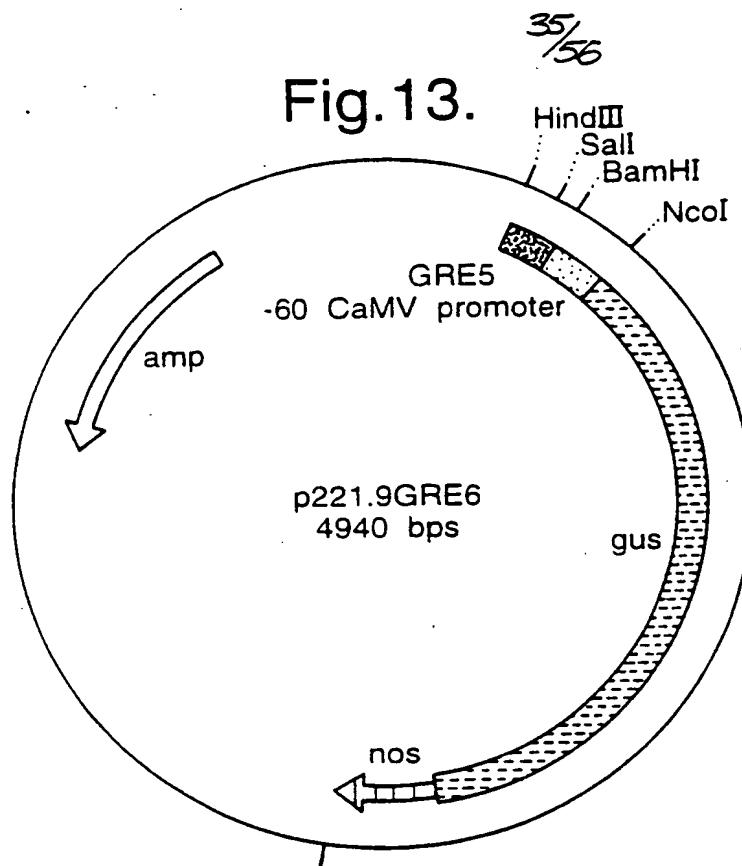
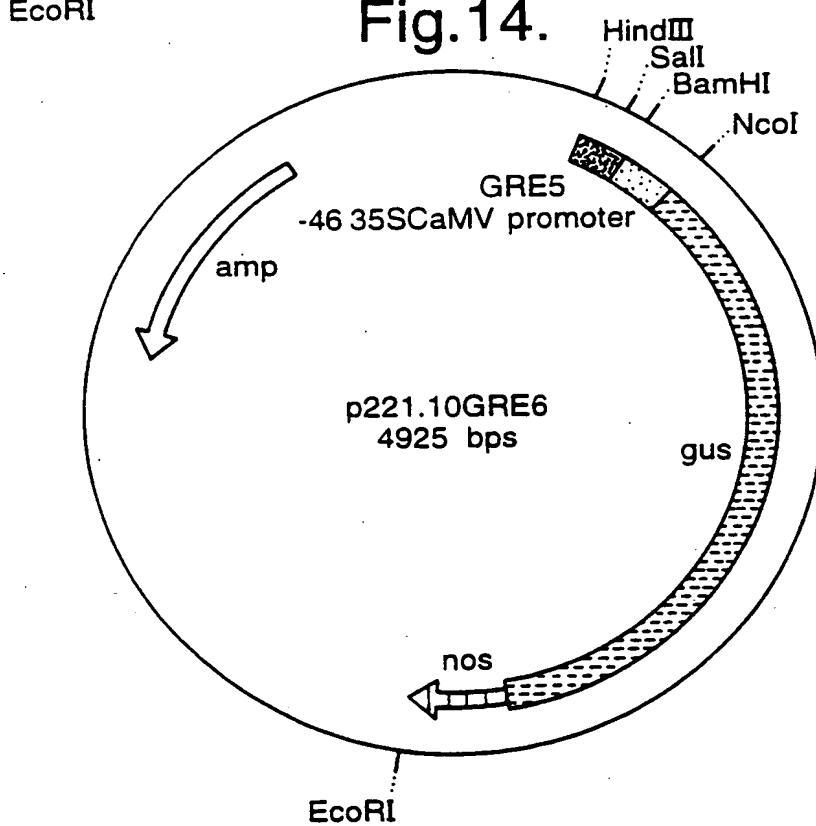


Fig.14.



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Fig.15.

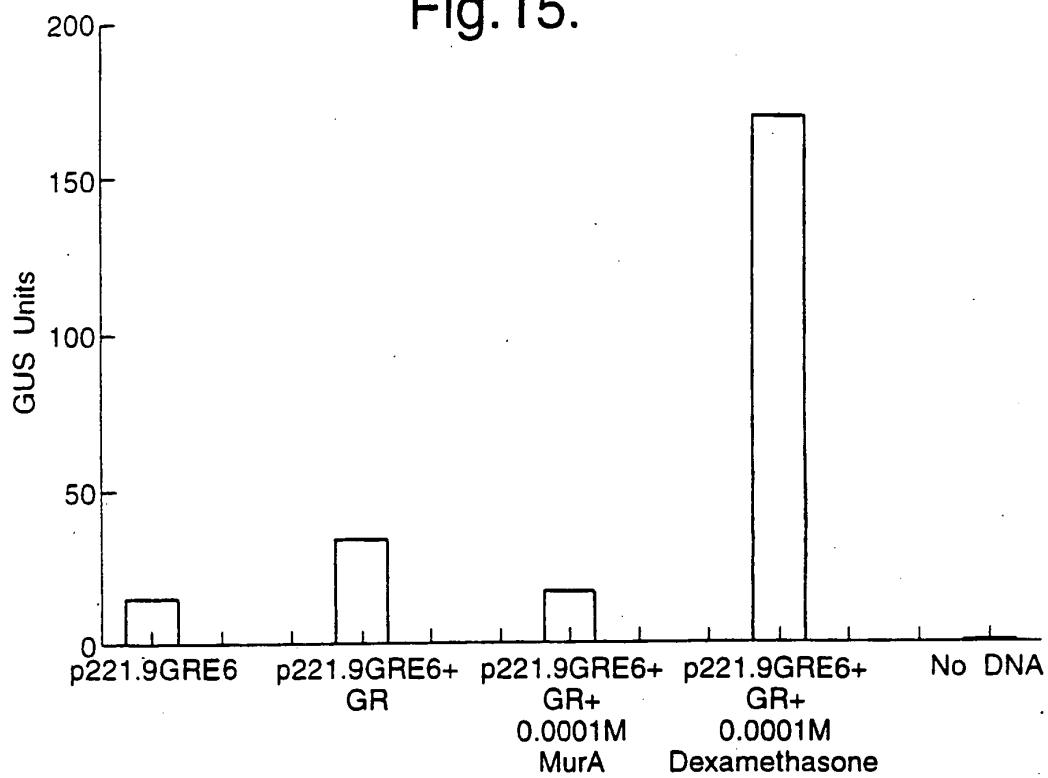
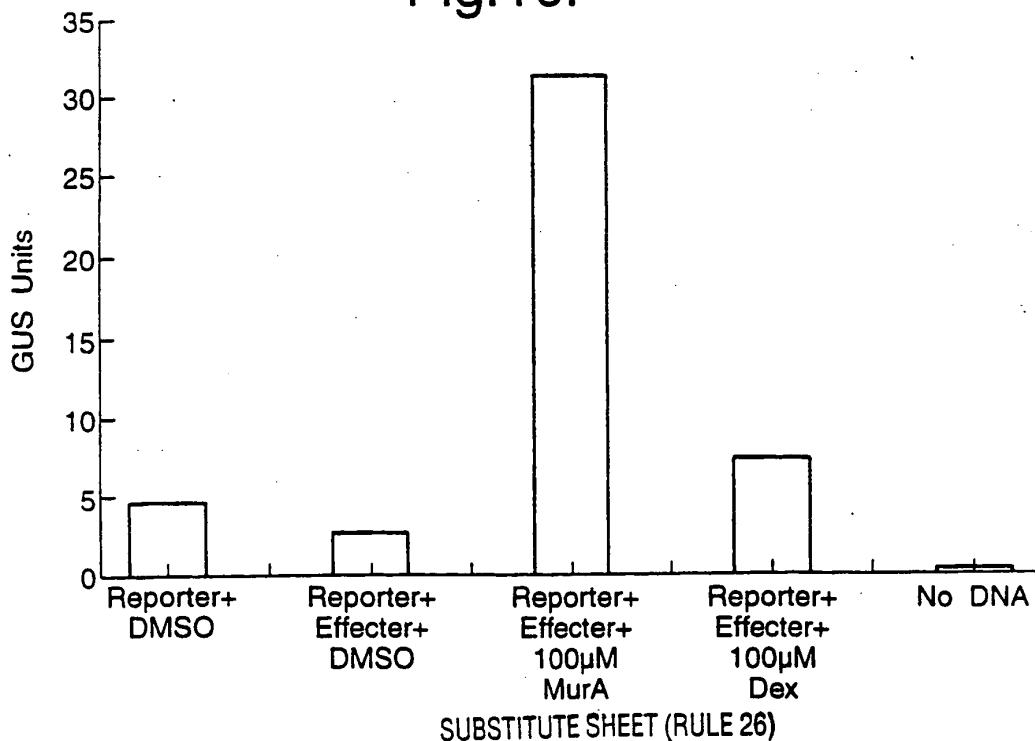


Fig.16.



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Fig.17.

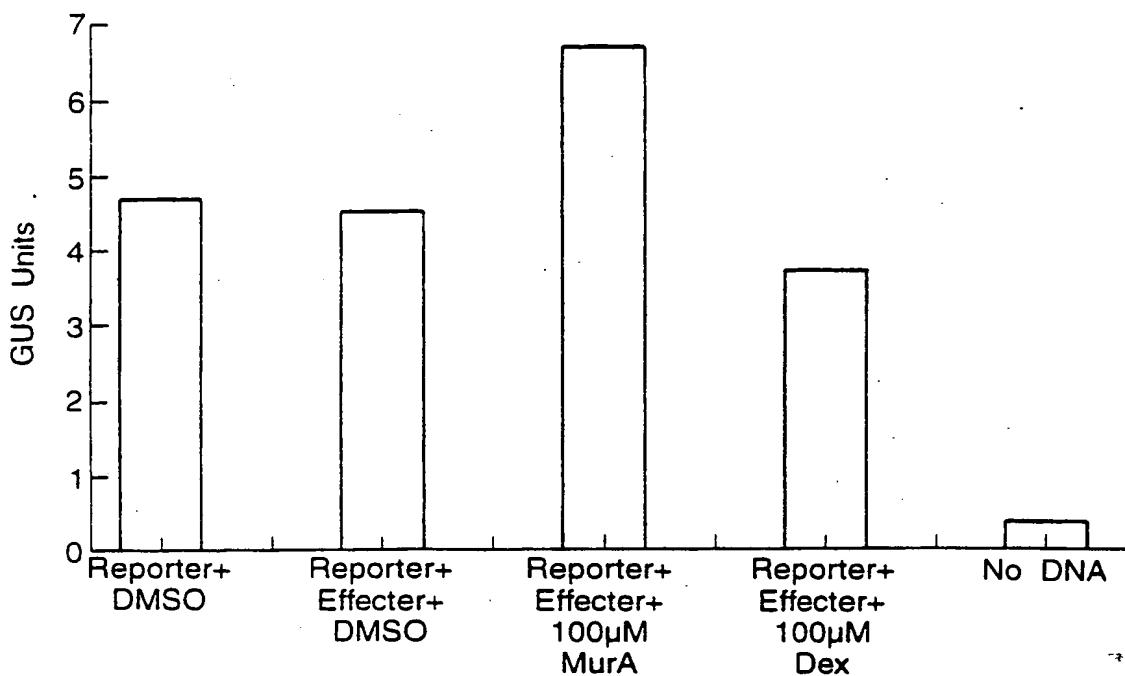
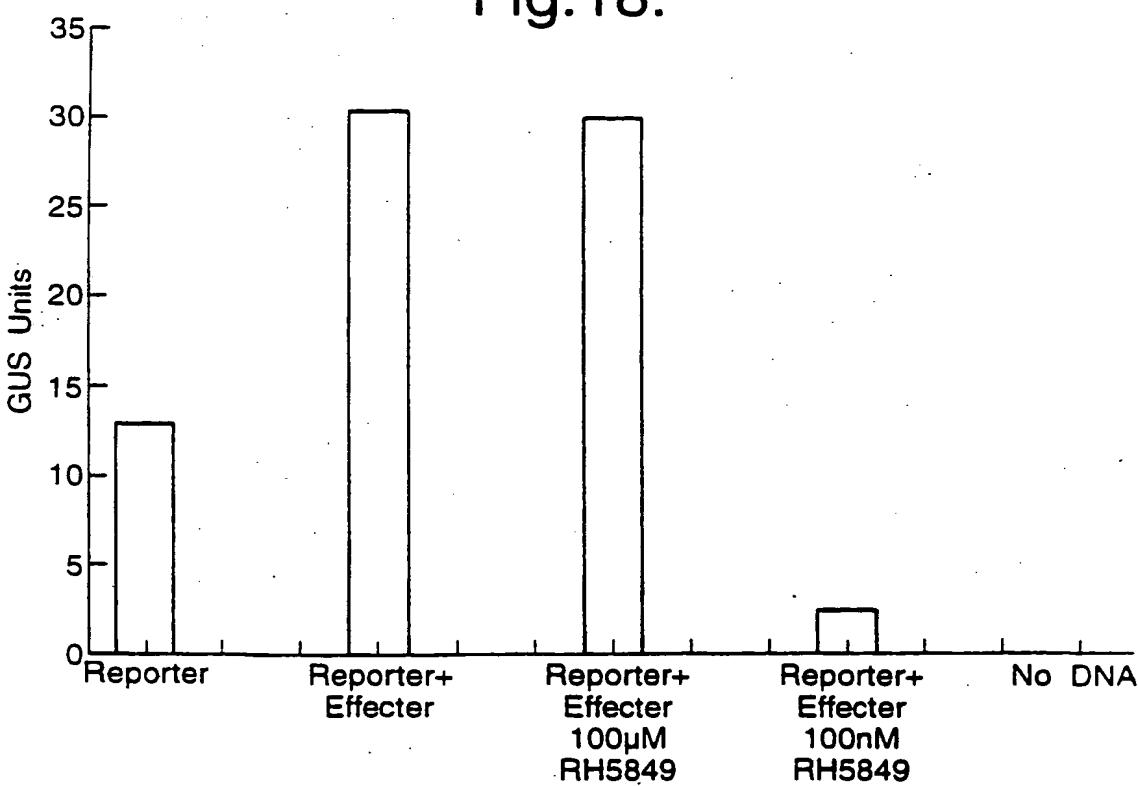


Fig.18.



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Fig.19.

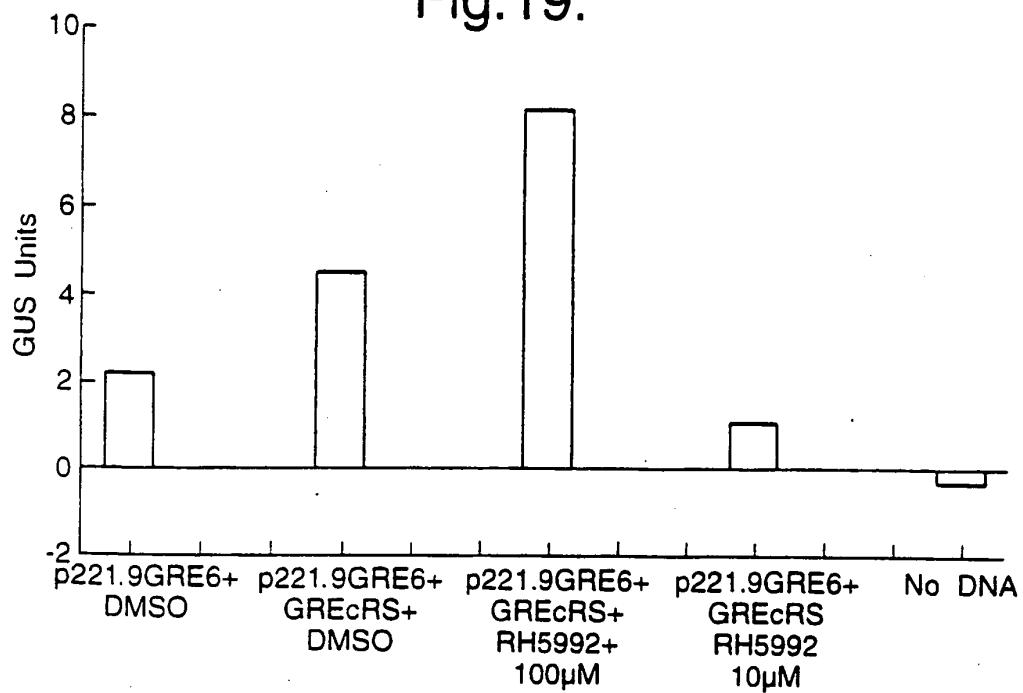


Fig.20.

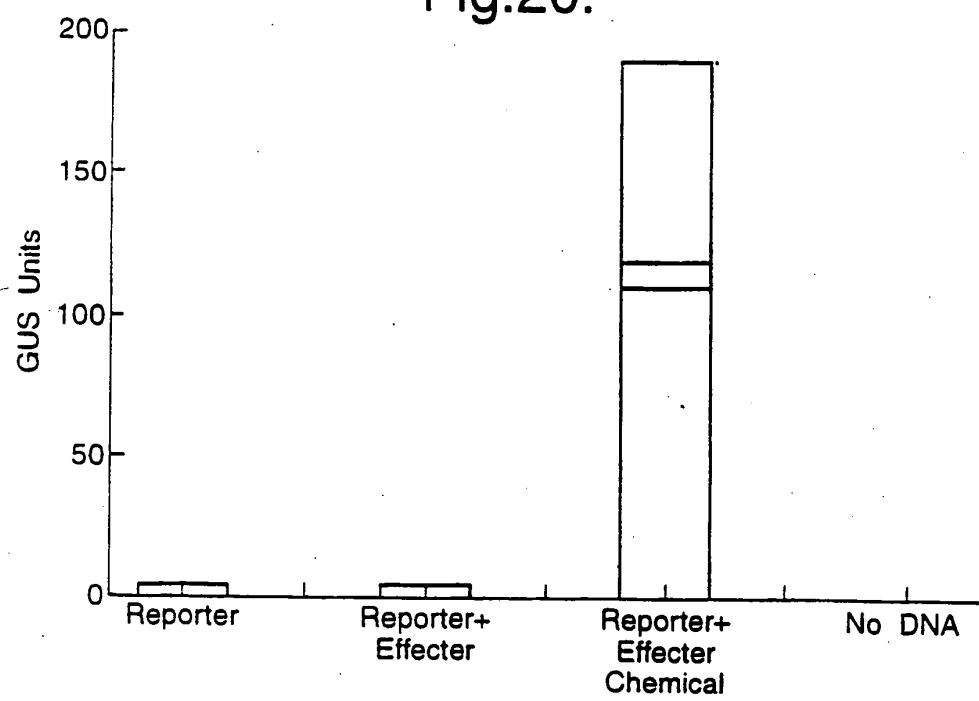
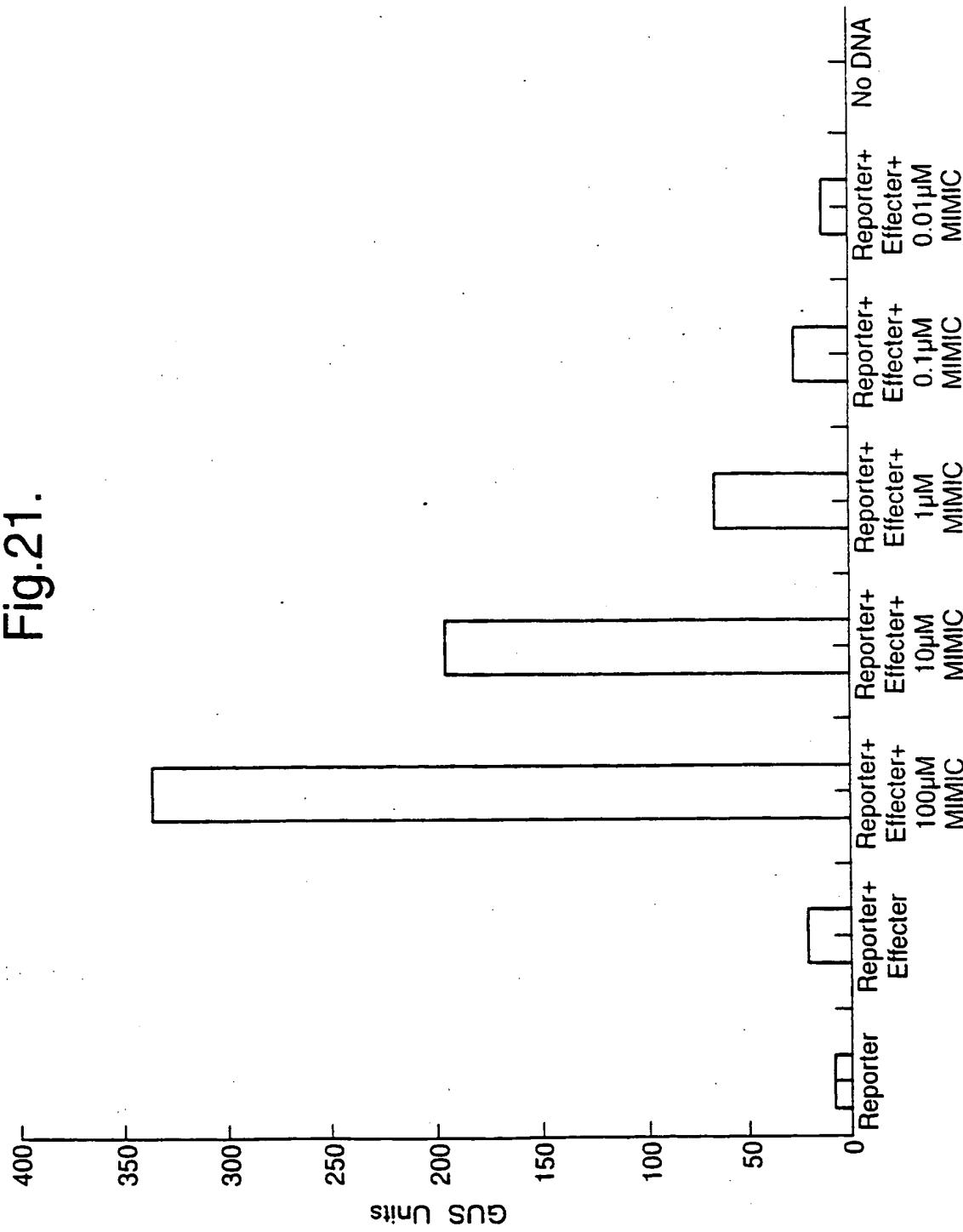


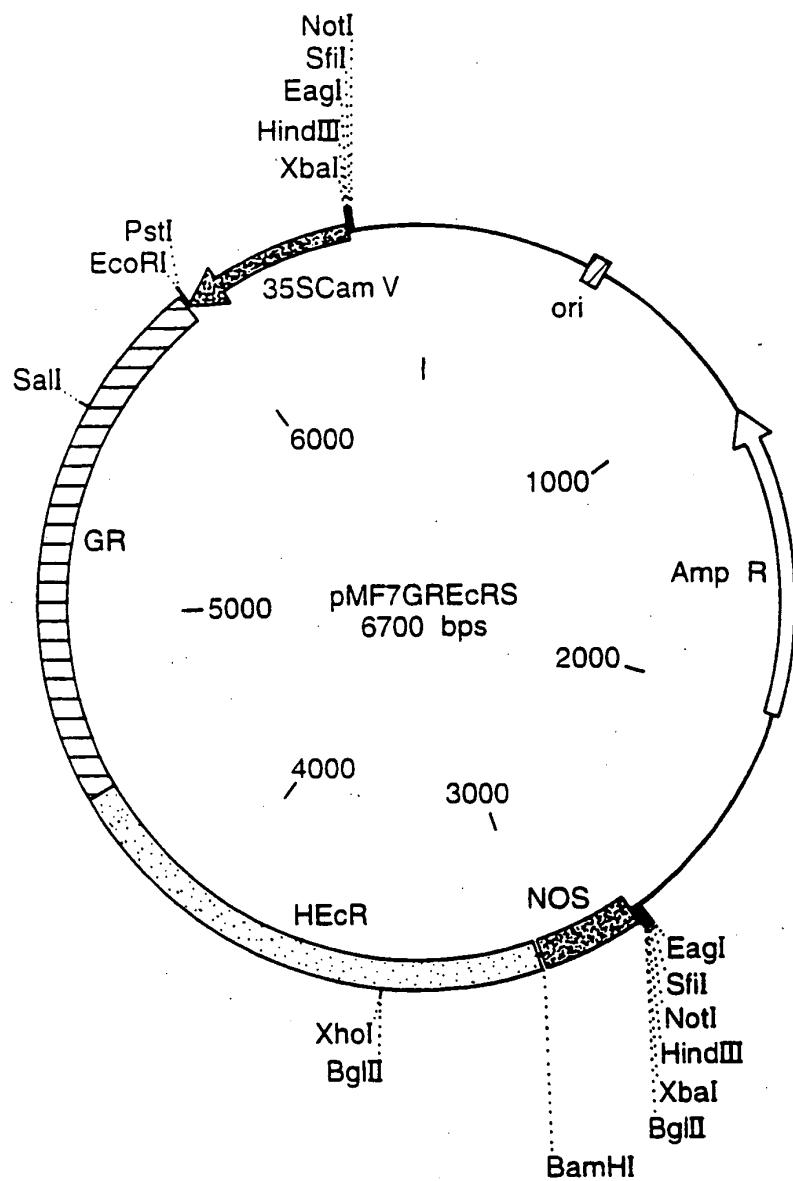
Fig.21.



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40
56

Fig.22.



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Fig.23.

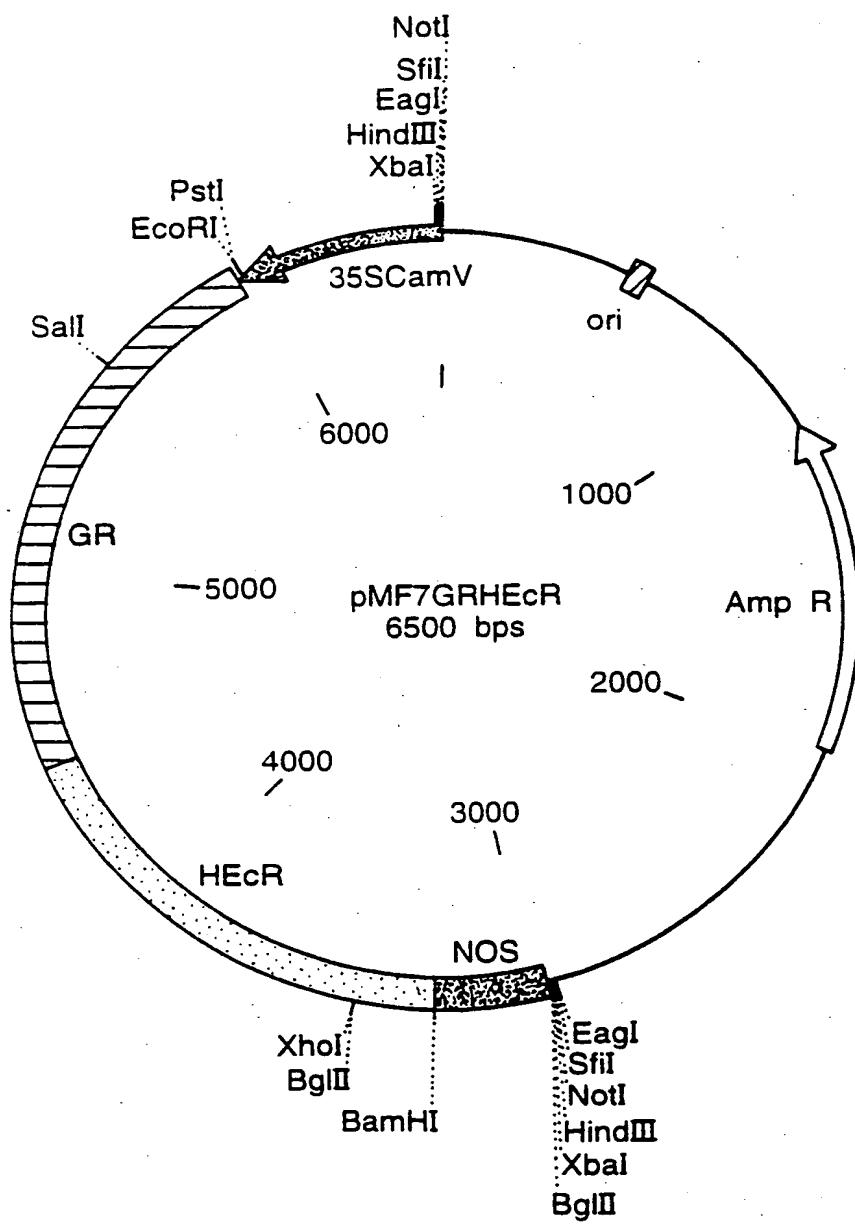


Fig.24.

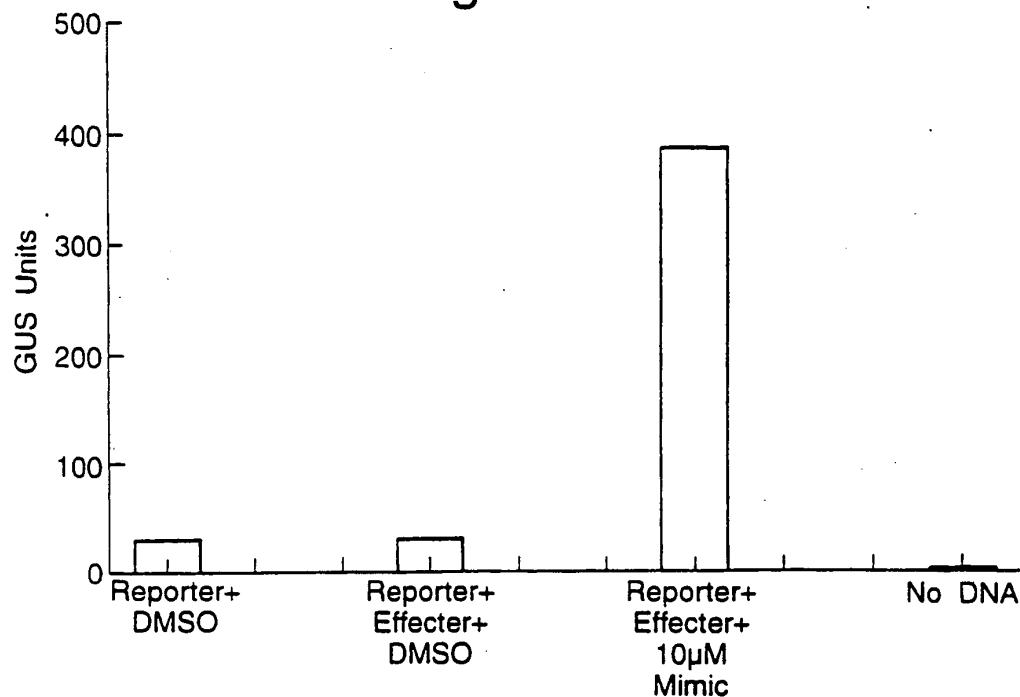
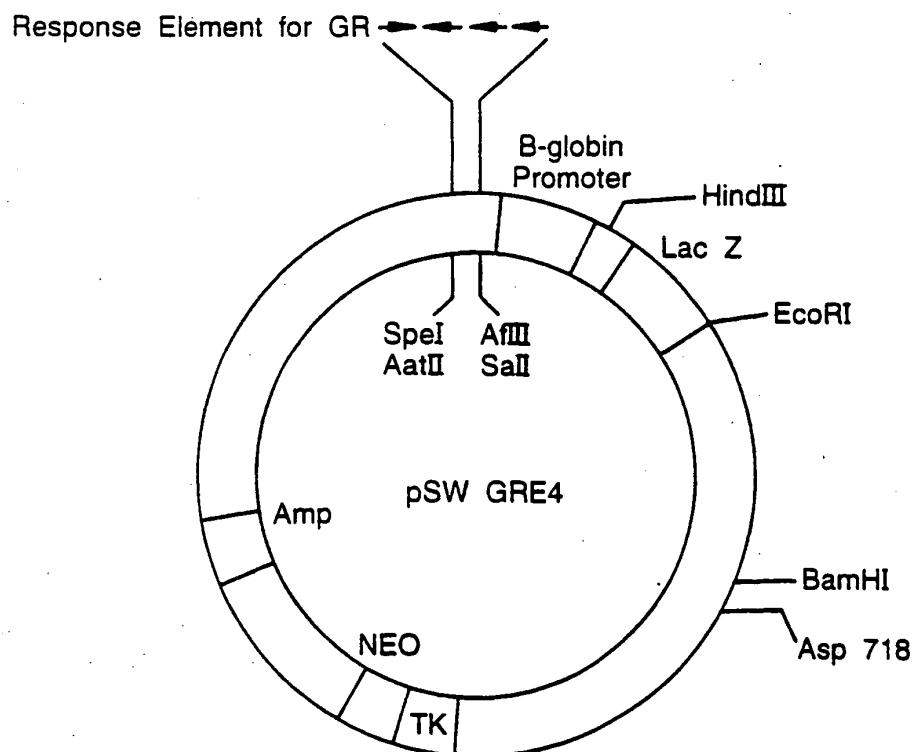


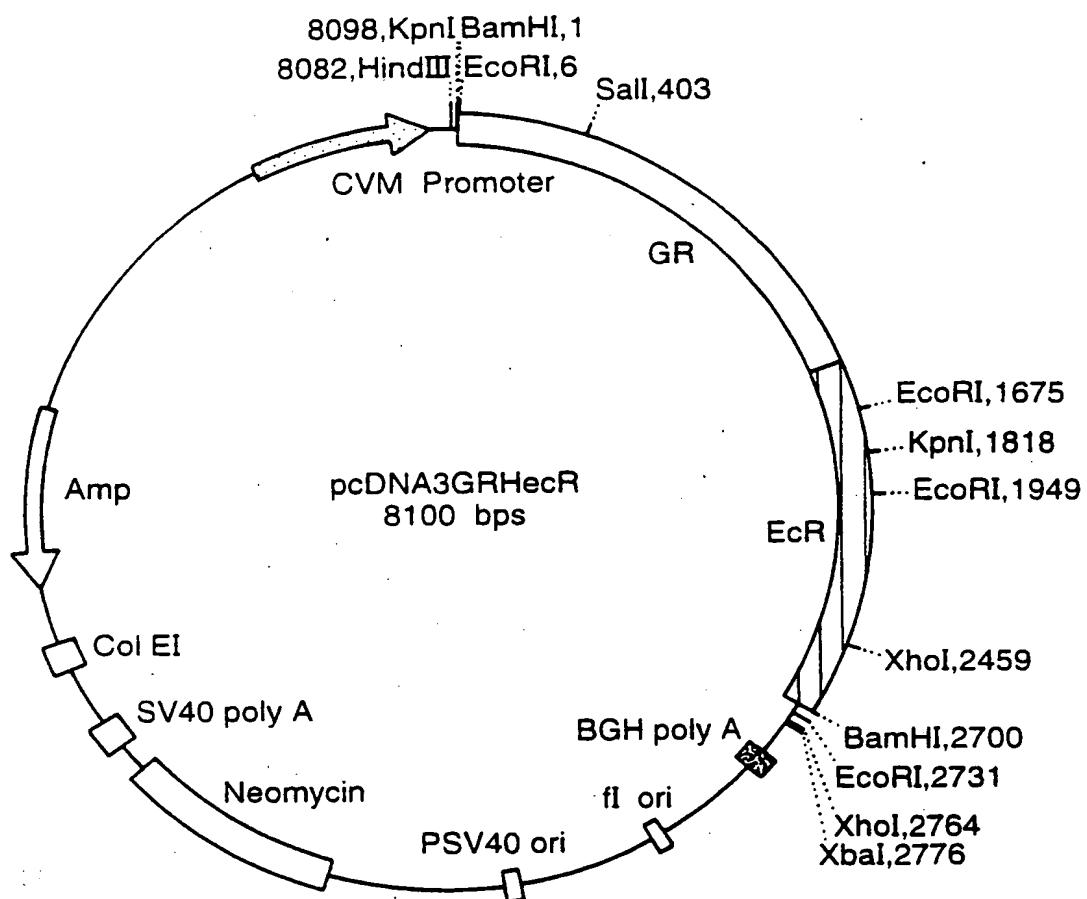
Fig.26.



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Fig.25.



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56

Fig.27.

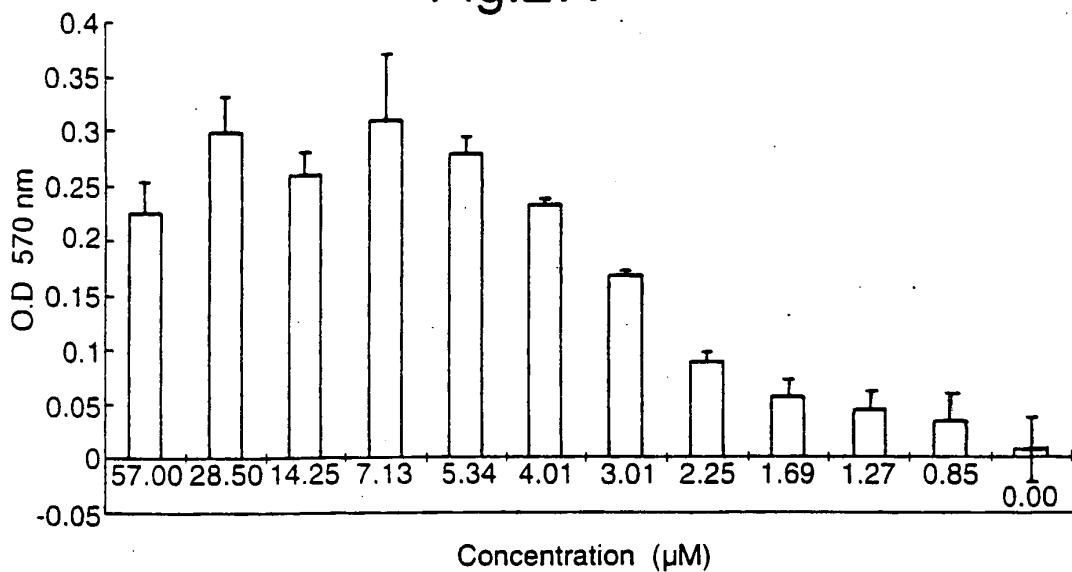
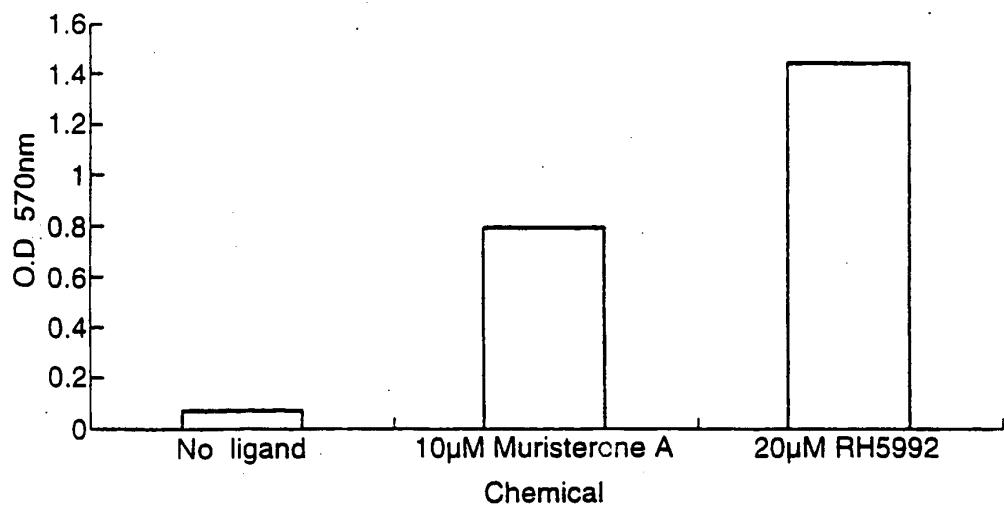


Fig.28.



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Fig.29.

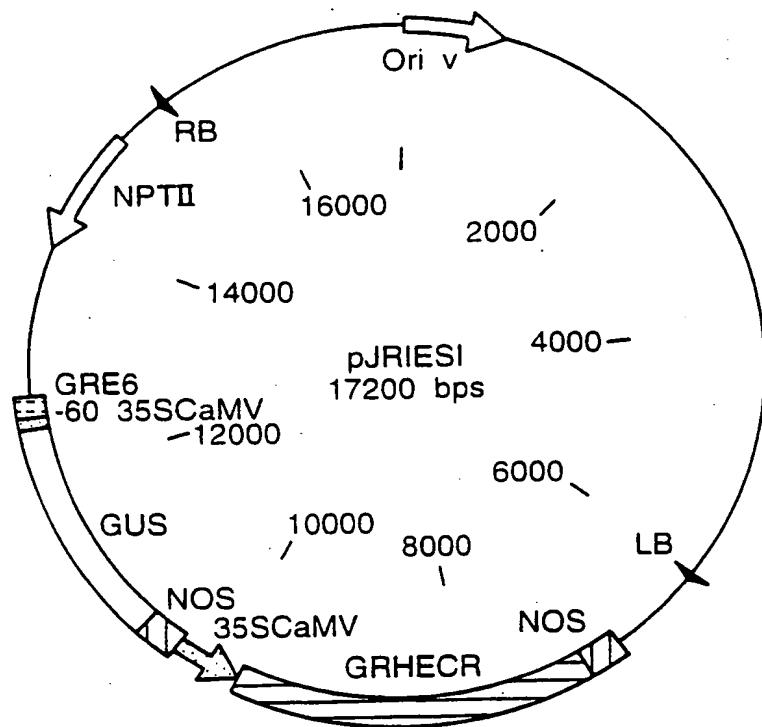
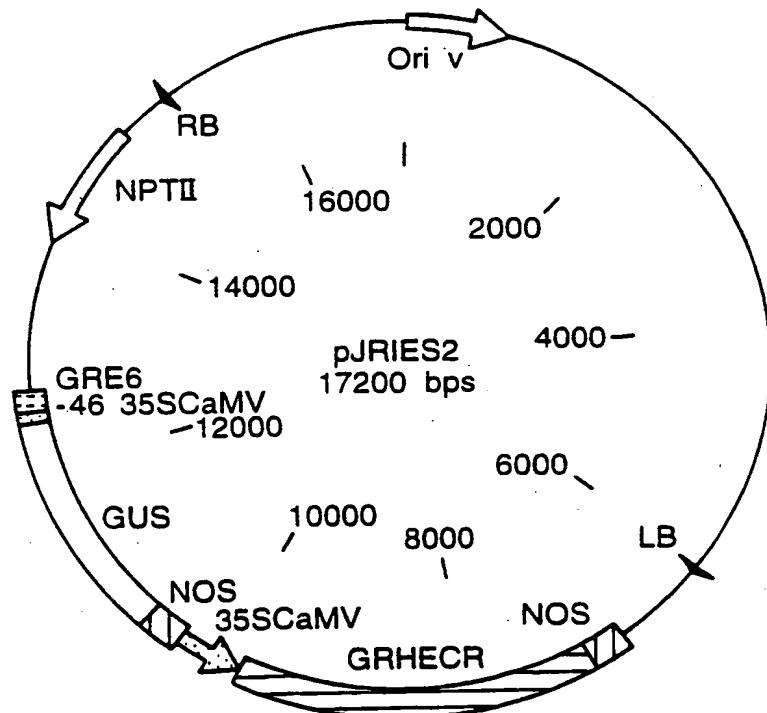


Fig.30.



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Fig.31.

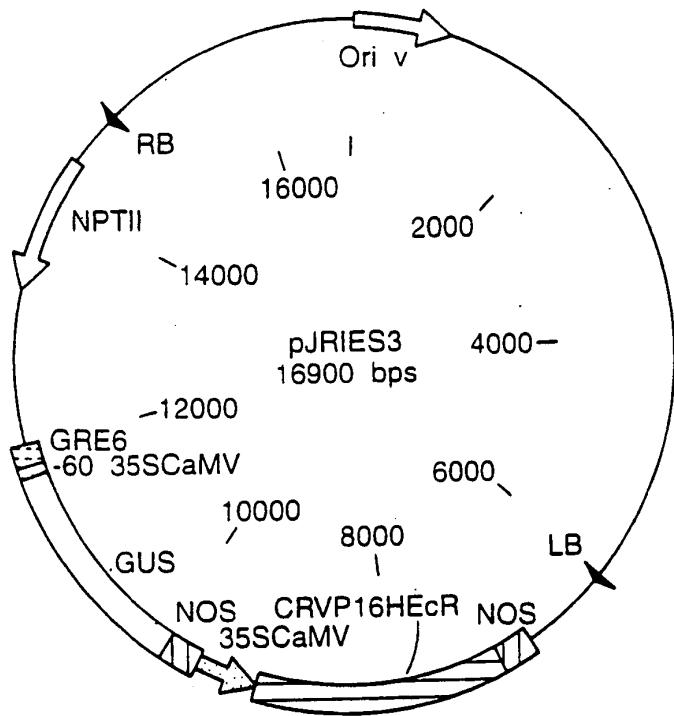


Fig.32.

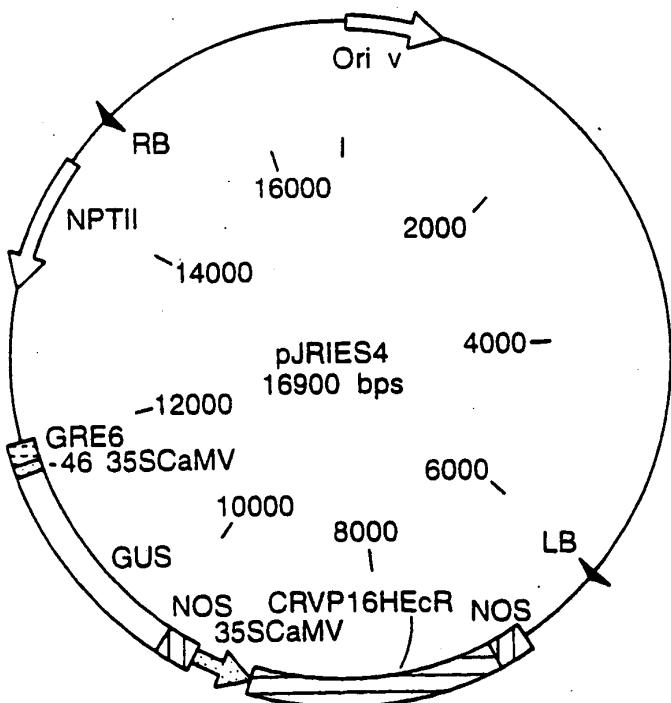


Fig.33.

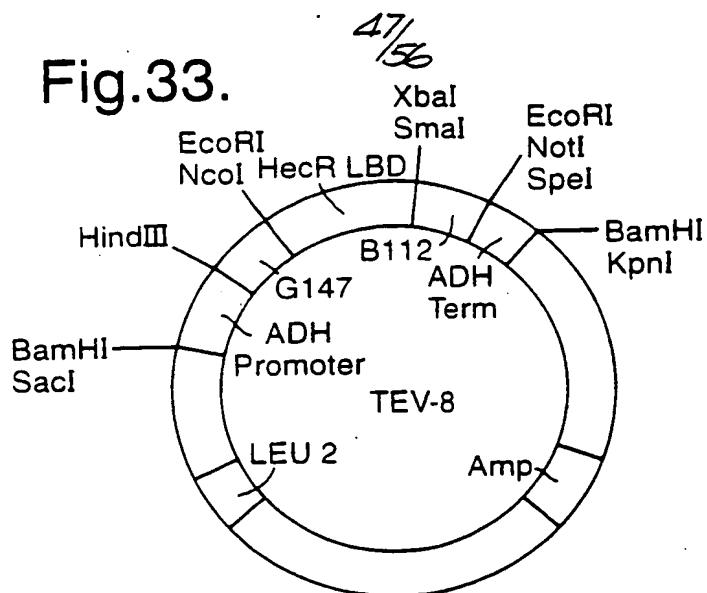


Fig.34.

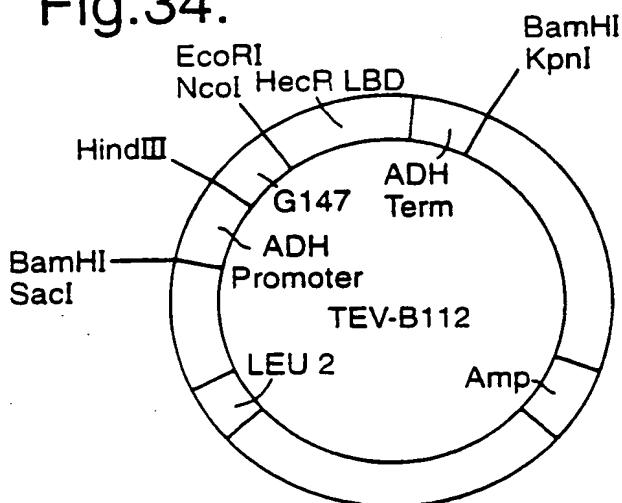
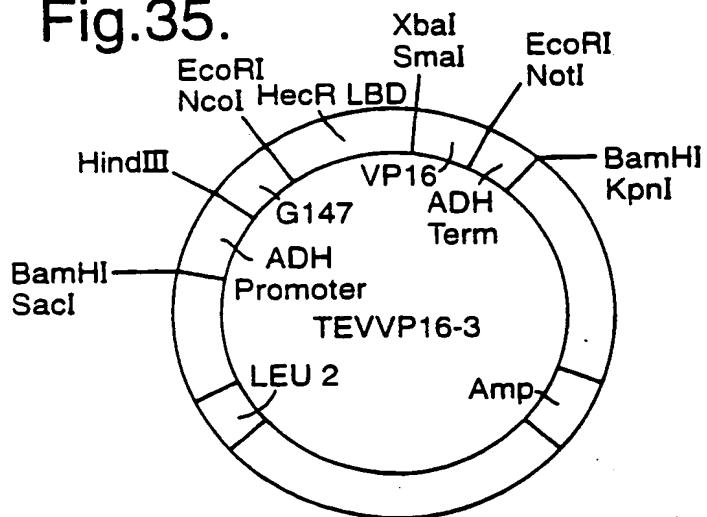


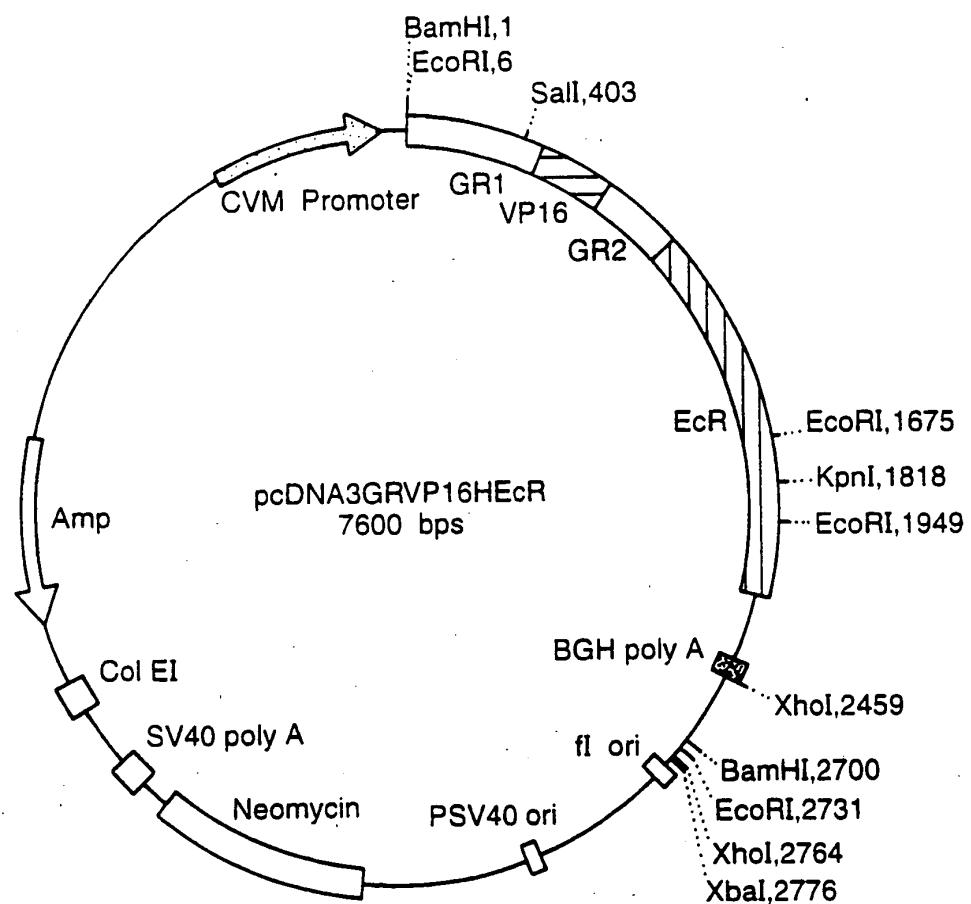
Fig.35.



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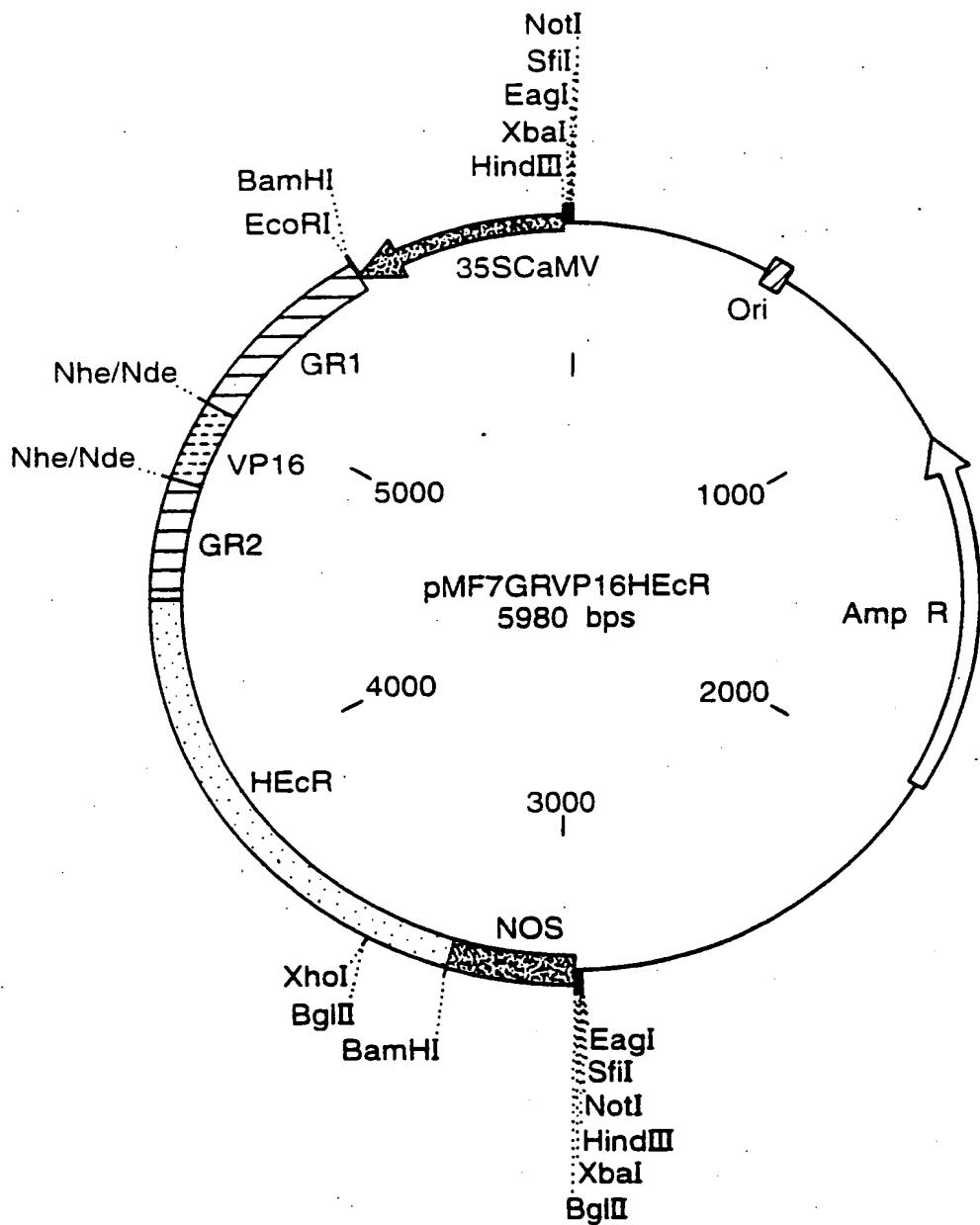
48
156

Fig.36.



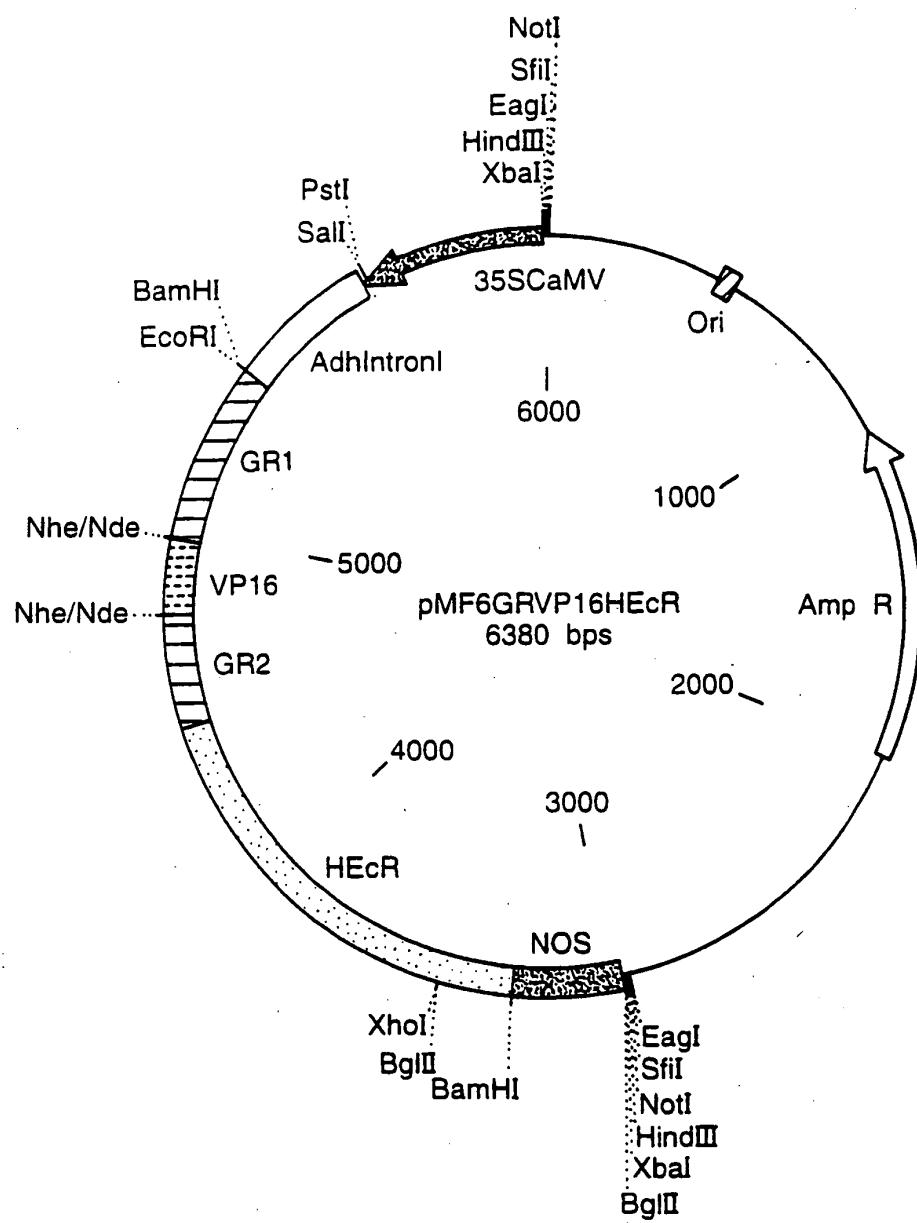
49
56

Fig.37.



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56

Fig.38.



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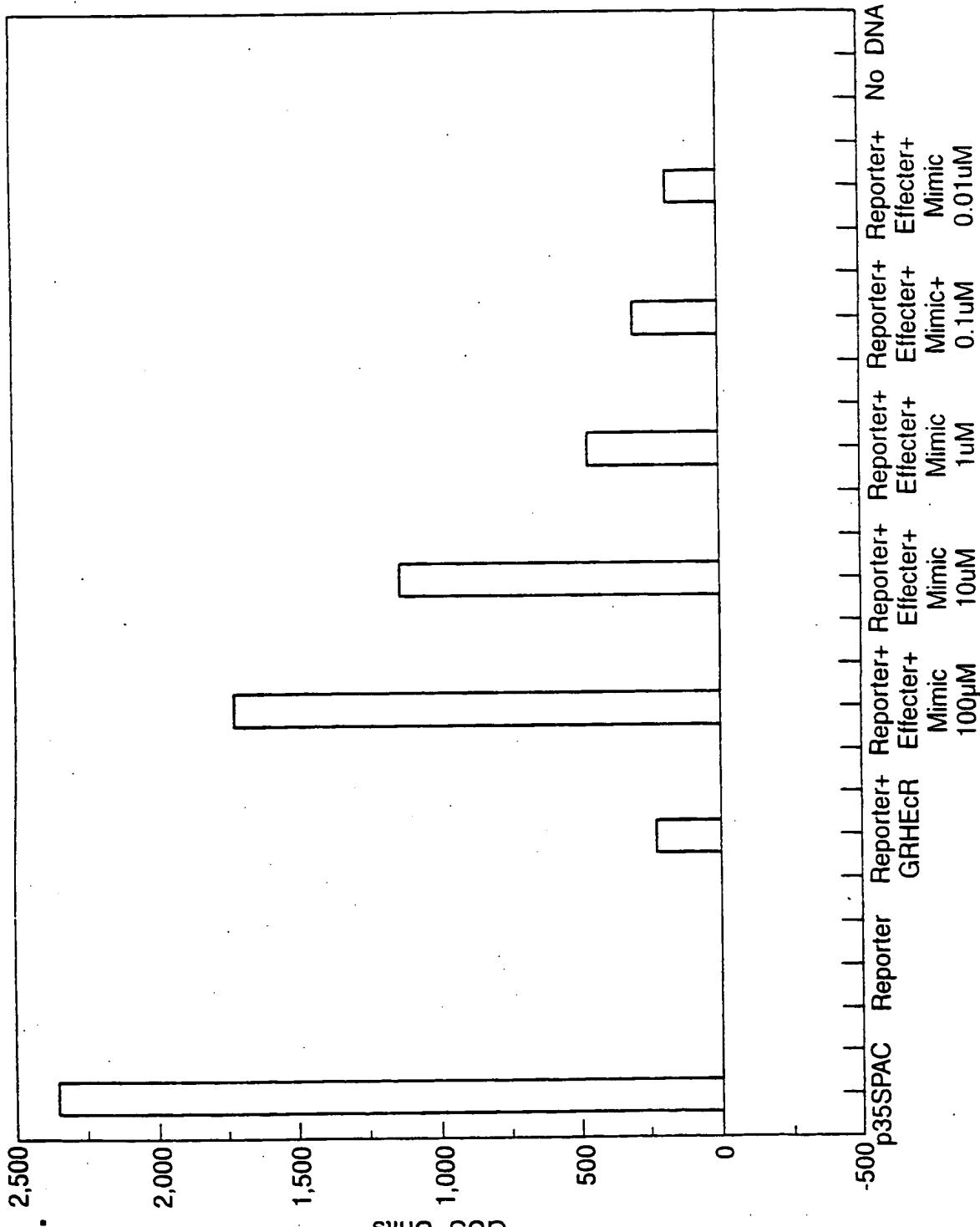


Fig.39. 2

Fig.40.

Spodoptera exigua DNA sequence.

Sequence ID 6

SPODOPTERA EXIGUA HINGE AND LIGAND BINDING DOMAINS

3 1	9 AGG GAG TGC GTG CCA GAA AAC CAG TGT GCA ATG AAA AGG TCC GGC CTC ACG CAC CAC GGT CTT RTG GTC ACA CGT TAC TTT TCC	15 AAA GAG AAA AAG GCA CAA AGG GAA AAA GAC AAG TTG CCA GTC AGT TTT CTC TTC CGT GTT TCC CTT CCT CCC ATT ATG CAG TGT GAT	21 91 ACA ACG ACA GTG GAT GAT CAC ATG CCT CCC ATT ATG CAG TGT GAT TGT TGC TGT CAC CTA CTA GTG TAC GGA GGG TAA TAC GTC ACA CTA	27 136 CCA CCG CCT CCA GAG GCC GCA AGA ATT CAC GAG GTG GTG CCA CGA GGT GGC GGA GGT CTC CGG CGT TCT TAA GTG CTC CAC CAC GGT GCT	33 181 TTC CTG AAT GAA AAG CTA ATG GAC AGG ACA AGG CTC AAG AAT GTG AAG GAC TTA CTT TTC GAT TAC CTG TCC TGT TCC GAG TTC TTA CAC	39 226 CCC CCT CAC TGC CAA CCA GAA GTC CTT AAT AGC GAG GCT GGT CTG GGG GGA GTG ACG GTT GGT CTT CAG GAA TTA TCG CTC CGA CCA GAC	45 271 GTA CCA AGA AGG CTA TGA ACA GCC ATC AGA GGA TCT AAA AAG CAT GGT TCT TCC GAT ACT TGT CGG TAG TCT CCT AGA TTT TTC
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Fig.40 i.

316 AGT CAC ACA GTC GGA TGA AGA CGA AGA GTC GGA CAT GCC GTT
 TCA GTG TGT CAG CCT ACT TCT GCT TCT TCT TCT CAG CCT GTA CGG CAA
 361 CCG TCA GAT CAC CGA GAT GAC GAT CCT CAC AGT GCA GCT CAT TGT
 GGC AGT CTA GTG GCT CTA CTG CTA CGA GTG TCA CGT CGA GTA ACA
 406 TGA ATT CGC TAA GGG CCT ACC AGC GTT CGC AAA GAT CTC ACA GTC
 ACT TAA GCG ATT CCC GGA TGG TCG CAA GCG TTT CTA GAG TGT CAG
 451 GGA TCA GAT CAC ATT ATT AAA GGG CTG TTC GAG TGA GGT GAT GAT
 CCT AGT CTA GTG TAA TAA TTT CGG GAC AAG CTC ACT CCA CTA CTA
 496 GTT GCG AGT AGC TCG GCG GTA CGA CGC GGC GAC AGA CAG CGT GTT
 CAA CGC TCA TCG AGC CGC CAT GCT GCG CCG CTG TCT GTC GCA CAA
 541 GTT CGC CAA CCA CCA GGC GTA CAC CCG CGA CAA CTA CCG CAA GGC
 CAA CGC GTT GTT GGT CGT CGC CAT GTG GGC GCT GTT GAT GGC GTT CCG
 586 AGG CAT GGC CTA CGT CAT CGA GGA CCT GCT GCA CTT CTG CCG GTG
 TCC GTA CCG GAT GCA GTA GCT CCT GGA CGA CGT GAA GAC GGC CAC
 631 CAT GTA CTC CAT GAT GGA TAA CGT CCA CTA TGC ACT GCT CAC
 GTA CAT GAG GTA CTA CTC ATT GCA GGT GAT ACG TGA CGA GTG
 676 TGC CAT CGT CAT TTT CTC AGA CCG ACC CGG GCT TGA GCT AAC CCT
 ACG GTA GCA GTA AAA GAG TCT GGC TGG GCC CGA ACT CGA TTG GGA
 721 GTT GGT GGA GGA GAT CCA GAG ATA TTA CCT GAA CAC GCT GCG GGT
 CAA CCA CCT CTC GGT CTC TAT AAT GGA CTT GTG CGA CGC CCA

SUBSTITUTE SHEET (RULE 26)

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766	GTA	CAT	CCT	GAA	CCA	GAA	CAG	TCG	GTC	GCC	GTG	CTG	CCC	TGT	CAT
	CAT	GTA	GGA	CTT	GGT	CTT	GTC	AGC	AGC	CGG	CAC	GAC	GGG	ACA	GTA
811	CTA	CGC	TAA	GAT	CCT	CGG	CAT	CCT	GAC	GGG	GCT	GCG	GAC	CCT	GGG
	GAT	GGC	ATT	CTA	GGA	GCC	GTA	GGA	CTG	CCT	CGA	CGC	CTG	GGA	CCC
856	CAT	GCA	GAA	CTC	CAA	CAT	GTG	CAT	CTC	ACT	CAA	GCT	GAA	GAA	CAG
	GTA	CGT	CTT	GAG	GTT	GTA	CAC	GTA	GAG	TGA	GTT	CGA	CTT	CTT	GTC
901	GAA	CGT	GCC	GCC	GTT	CTT	CGA	GGA	TAT	CTG	GGA	CGT	CCT	CGA	GTA
	CTT	GCA	CGG	CGG	CAA	GAA	GCT	CCT	ATA	GAC	CCT	GCA	GGA	GCT	CAT
946	AAA														

Total number of bases is: 948.

Sequence I.D. 7

Fig.41.

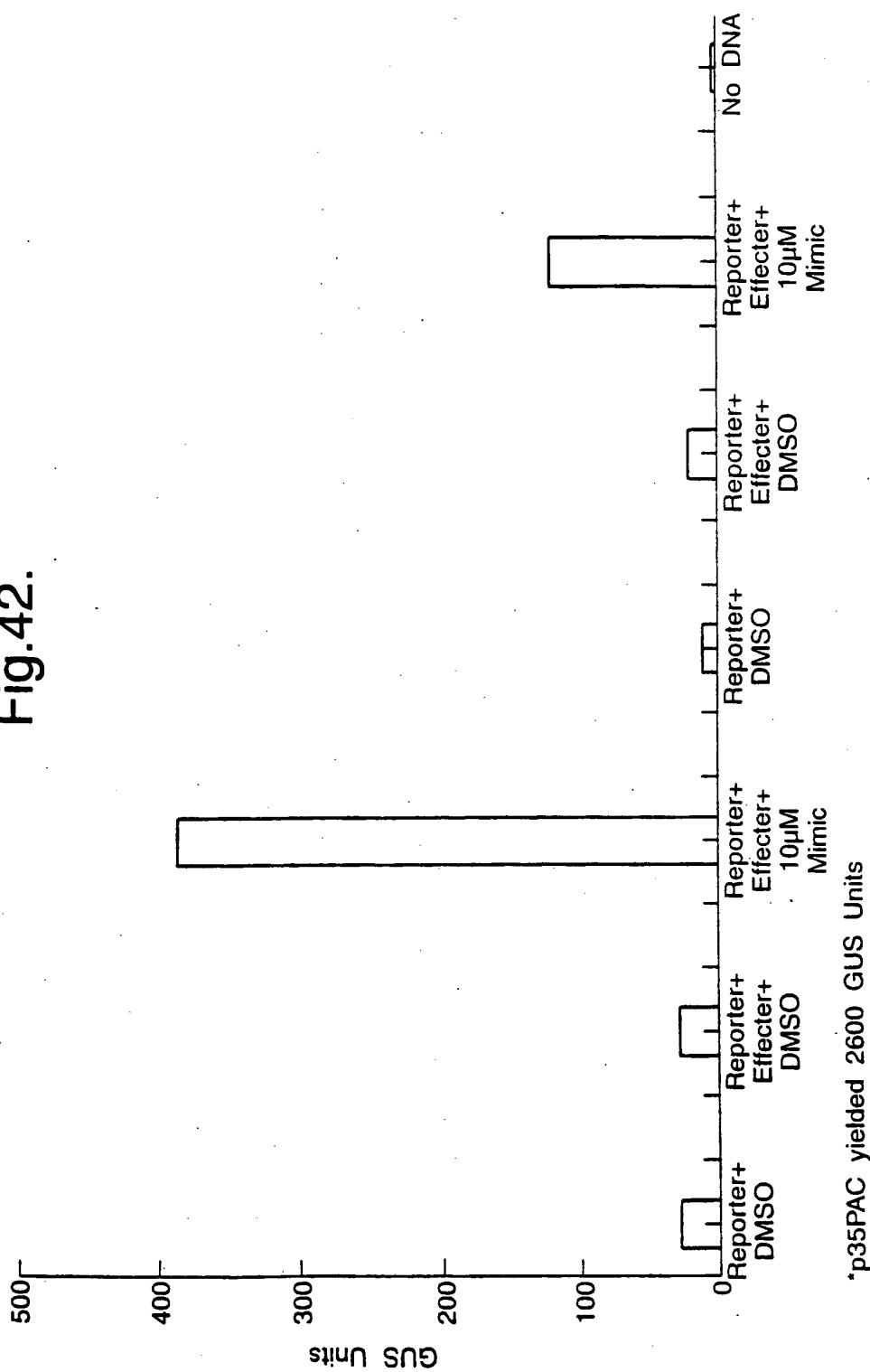
Sequence comparison between *Heliothis 19R* clone and *SECR* *Taq* clone

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HECR	RPECVVPENQCAMRKKEKKAAQREKDKLPPVSTTTVDDHMPPIQMCDPPPPEAARILECVQ
SECR	RPECVVPENQCAMRKKEKKAAQREKDKLPPVSTTTVDDHMPPIQMCDPPPPEAARI
HECR	HEVVPRFLNEKLMEQNRLKVNPPPLTANQKSLIARLVWYQEGYEQPSEEDLKRVQTQSD
SECR	HEVVPRFLNEKLME TRL <u>RNV</u> PPPLTANQKSLIARLVWYQEGYEQPSEEDLKRVQTQSD
HECR	EDDEDSDMPFRQITITEMTILTQLIVEFAKGGLPGFAKISQSDQITLLKACSSSEVMLR
SECR	EDEEEESDMPFRQITITEMTILTQLIVEFAKGGLPAFAKISQSDQITLLKACSSSEVMLR
HECR	VARRYDAATDSVLFANNQAYTRDNYRKAGMAYVIEDLLHFRCMYSMMDNHYALL
SECR	VARRYDAATDSVLFANNQAYTRDNYRKAGMAYVIEDLLHFRCMYSMMDNHYALL
HECR	TAIVIFSDRPGLEQPLLVEIQRYYLNTLRVYILNQNSASPRGAVIFGEILGILTEI
SECR	TAIVIFSDRPGLE <u>LLV</u> VEIQRYYLNTLRVYILNQNSRS <u>SPCCPV</u> YAKILGILTEL
HECR	RTLGMQNSNMCISLKLKKRKLPPFLEEIDWDV
SECR	RTLGMQNSNMCISLKLKKRKLPPFEE <u>IDWDV</u>

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Fig.42.



INTERNATIONAL SEARCH REPORT

Inte
n Application No
PCT/GB 96/01195

A. CLASSIFICATION OF SUBJECT MATTER				
IPC 6 C12N15/12	C12N15/85	C12N15/62	C07K14/72	C07K19/00
C12N5/10	A61K38/16			

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C07K C12N A01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,93 03162 (GENENTECH INC) 18 February 1993	4,5,44, 92-99
Y	see abstract; claims 1-27; figure 1	1,3, 8-43, 45-49, 51-91
X	---	4,5,44, 50,93-99
Y	WO,A,91 13167 (UNIV LELAND STANFORD JUNIOR) 5 September 1991 see abstract; claims 2,24 ---	2,3
		-/-

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

* Special categories of cited documents :

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

- *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
- *&* document member of the same patent family

Date of the actual completion of the international search

9 August 1996

Date of mailing of the international search report

19.08.96

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentiaan 2
NL - 2280 HV Rijswijk
Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl.
Fax (+ 31-70) 340-3016

Authorized officer

Gurdjian, D

INTERNATIONAL SEARCH REPORT

Int'l Application No
PCT/GB 96/01195

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CELL, OCT 4 1991, 67 (1) P59-77, UNITED STATES, XP002010069 KOELLE MR ET AL: "The Drosophila EcR gene encodes an ecdysone receptor, a new member of the steroid receptor superfamily." see the whole document	4,5
Y		1-3, 8-43, 45-49, 51-92
X	INSECT BIOCHEM MOL BIOL, JAN 1993, 23 (1) P115-24, ENGLAND, XP002010070 IMHOF MO ET AL: "Cloning of a Chironomus tentans cDNA encoding a protein (cEcRH) homologous to the Drosophila melanogaster ecdysteroid receptor (dEcR)." see the whole document	4,5
X	INSECT BIOCHEM MOL BIOL, JAN 1995, 25 (1) P19-27, ENGLAND, XP002010071 CHO WL ET AL: "Mosquito ecdysteroid receptor: analysis of the cDNA and expression during vitellogenesis." see the whole document	4,5,52, 53
Y	EP,A,0 615 976 (AMERICAN CYANAMID CO) 21 September 1994 see page 6, line 28 - line 32; claims 1-12; example 2	8-43, 45-49, 51-92
Y	EUR. J. ENTOMOL. (1995), 92(1), 333-40 CODEN: EJENE2; ISSN: 1210-5759, XP002010346 SMAGGHE, GUY ET AL: "Biological activity and receptor -binding of ecdysteroids and the ecdysteroid agonists RH-5849 and RH-5992 in imaginal wing discs of Spodoptera exigua (Lepidoptera: Noctuidae)" see page 336, paragraph 3 - page 337, paragraph 2	51-65
A	DEVELOPMENTAL GENETICS, 1995, 17, 319-330, XP002010345 KOTHAPALLI R ET AL: "CLONING AND DEVELOPMENTAL EXPRESSION OF THE ECDYSONE RECEPTOR GENE FROM THE SPRUCE BUDWORM, CHORISTONEURA-FUMIFERANA" see the whole document	1-5, 51-54
		-/-

INTERNATIONAL SEARCH REPORT

Inte
nal Application No
PCT/GB 96/01195

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	INSECT BIOCHEM. MOL. BIOL. (1994), 24(8), 763-73 CODEN: IBMBES; ISSN: 0965-1748, XP002010072 JINDRA, MAREK ET AL: "Isolation and developmental expression of the ecdysteroid-induced GHR3 gene of the wax moth <i>Galleria mellonella</i> " see the whole document --- US,A,5 424 333 (WING KEITH D) 13 June 1995 see column 150, paragraph 3 - paragraph 7; example 3 -----	1-5
A		97,98

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/GB 96/01195

Patent document cited in search report	Publication date	Patent family member(s)	Publication date	
WO-A-9303162	18-02-93	EP-A- 0598011 JP-T- 7501928	25-05-94	02-03-95
WO-A-9113167	05-09-91	AU-B- 1779295 AU-B- 7492291 CA-A- 2076386 EP-A- 0517805 US-A- 5514578	14-09-95	18-09-91
EP-A-0615976	21-09-94	CA-A- 2112445 JP-A- 6253849	01-07-94	13-09-94
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			03-06-93	21-12-89
			04-11-93	10-03-94
			04-04-90	16-11-94
			12-06-90	17-09-92
			21-12-89	07-10-93
			14-04-94	30-06-94
			20-12-89	16-11-94
			31-07-95	31-02-90
			31-01-95	10-06-94
			26-05-92	29-03-90
			04-02-88	04-11-92
			12-12-91	30-01-94
			20-01-88	16-07-96

INTERNATIONAL SEARCH REPORT

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. Claims Nos.: **98**
because they relate to subject matter not required to be searched by this Authority, namely:
Although this claim is directed partly to a method of treatment of the human/animal body the search has been carried out and based on the alleged effects of the compound/composition
2. Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

The additional search fees were accompanied by the applicant's protest.
 No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Int'l. Appl. No.: PCT/GB 96/01195

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US-A-5424333		IE-B- 59964	04-05-94
		JP-B- 7098806	25-10-95
		JP-A- 63023866	01-02-88
		KR-B- 9505199	19-05-95
		AU-B- 602505	18-10-90
		AU-B- 6926687	03-09-87
		CA-A- 1295618	11-02-92
		EP-A- 0234944	02-09-87
		ES-T- 2032818	16-07-96
		KR-B- 9410277	22-10-94
		AU-B- 599970	02-08-90
		AU-B- 7147287	31-03-88
		CA-A- 1331189	02-08-94
		DE-A- 3783111	28-01-93
		EP-A- 0261755	30-03-88
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